The Mutation Frequency of *Vibrio cholerae*
A Comparison study of Planktonic and Biofilm Cells

Diplomarbeit

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Abstract

The pathogenic opportunist and causative agent of the endemic disease cholera *Vibrio cholerae* naturally occurs in two distinct forms, in a single cell state and in so-called biofilms, consisting of cells in a polysaccharide matrix. It was observed that during biofilm development an initially geno- and phenotypically homogenous population of *V. cholerae* cells diversifies and adapts to the biofilm environment, leading to a higher phenotypic diversity. This study investigates if the phenotypic diversity is due to genotypic differences, by focusing on one aspect of mutagenesis, the spontaneous mutation of cells. Therefore, mutation frequencies were determined using the lacI repressor as a mutation trap. The genetically-engineered construct, transformed into the bacteria's chromosome via natural transformation upon induction of natural competence by growth on chitin, enabled the examination of evolutionary processes in real-time on a molecular level. The mutated cells thereby arising, were localized in the biofilm by confocal laser scanning microscopy. The mutation frequency of harvested biofilm cells was established and compared to the frequency determined for planktonic cells. In a parallel approach, mutation frequencies were estimated by measuring the spontaneous acquisition of antibiotic resistance to streptomycin, spectinomycin or nalidixic acid. The study indicates that the mutation frequencies of both cell states are within the same order of magnitude, i.e. approximately one in a million cells mutates. However, the results imply that, while posing several challenges, a determination of the mutation rate instead of the mutation frequency would be more conclusive.

Kurzfassung

Das Pathogen *Vibrio cholerae* ist der Erreger der Cholera. Dieses Bakterium kommt in der Natur in zwei distinkten Formen vor, als Einzellzelle und in so genannten Biofilmen, die aus Zellen in einer Polysaccharidmatrix bestehen. Es wurde beobachtet, dass sich während der Biofilmentwicklung eine ursprünglich geno- und phenotypisch homogene Population von *V. cholerae* Zellen verändert und an die neue Biofilmumgebung anpasst, was zu einer höheren phenotypischen Diversität im Biofilm führt. Diese Arbeit untersucht, ob Unterschiede im Genotyp diese phenotypische Diversität hervorrufen, indem sie sich auf einen Aspekt der Mutagenese, die spontane Mutation von Zellen, konzentriert. Zu diesem Zweck wurden Mutationsfrequenzen mithilfe eines genetischen Konstrukts, das auf dem lacI Repressor beruht und die Echtzeitbeobachtung von evolutionären Prozessen auf der molekularen Ebene erlaubt, bestimmt. Das Konstrukt wurde via natürliche Transformation nach Induktion von natürlicher Kompetenz durch Wachstum auf Chitin in das Chromosom des Bakteriums eingefügt. Die mutierten Zellen konnten mithilfe von Konfokaler Laser Scanning Mikroskopie im Biofilm lokalisiert werden. Die Mutationsfrequenz der geernteten Biofilmzellen wurde ermittelt und mit der Frequenz der planktonischen Zellen verglichen. In einem parallelen Ansatz dazu wurden Mutationsfrequenzen über den spontanen Erwerb von Antiobiotikaresistenz gegen Streptomycin, Spectinomycin und Nalidixinsäure bestimmt. Diese Arbeit zeigt, dass die Mutationsfrequenzen mit ungefähr einer mutierten Zelle in einer Million Zellen bei Einzelzellen wie auch Biofilmzellen in der gleichen Größenordnung liegen. Die Resultate deuten jedoch darauf hin, dass eine Bestimmung der Mutationsrate anstelle der Mutationsfrequenz aussagekräftiger wäre.

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Table of contents

1 Introduction 1
1.1 Microbial biofilms 1
1.2 *Vibrio cholerae* 2
1.3 Evolution 4
1.4 Goals 4
2 Material and methods 6
2.1 Bacterial strains, plasmids and oligonucleotides 6
2.2 Media 6
2.3 Growth conditions 7
2.3.1 Planktonic *E. coli* cultures 7
2.3.2 Planktonic *V. cholerae* cultures 8
2.3.3 Preparation of competent *E.coli* DH5α-λpir cells 8
2.4 Strain constructions in *V. cholerae* 8
2.4.1 *In silico* design of cloning experiments 9
2.4.2 DNA Amplification by Polymerase Chain Reaction (PCR) 9
2.4.3 Agarose gel electrophoresis 10
2.4.4 DNA extraction from agarose gels 11
2.4.5 Purification of DNA from solution 11
2.4.6 Enzymatic Restriction 11
2.4.7 Vector dephosphorylation 12
2.4.8 Ligation 12
2.4.9 Transformation of chemically competent *E. coli* cells with plasmids 12
2.4.10 Colony PCR 13
2.4.11 Freezer stocks 13
2.4.12 Plasmid purification 13
2.4.13 Sequencing and sequence analysis 14
2.4.14 Natural transformation on crab shell surfaces 14
2.4.15 *V.cholerae* colony PCR 15
2.5 Experiments with planktonic cultures of *V.cholerae* 16
2.5.1 Growth curves 16

2.5.2 Mutation frequency determination 16
2.5.3 Determination of acquisition of antibiotic resistance through point mutations 17
2.6 Experiments with *V.cholerae* biofilm cultures 17
2.6.1 Biofilm setup 17
2.6.2 *V. cholerae* biofilm cultures 18
2.6.3 Harvesting biofilm cells 19
2.6.4 Cell staining with the SYTO 63 red fluorescent nucleic acid stain 20
2.6.5 Image acquisition and processing 20
3 Results 22
3.1 Strain constructions in *V. cholerae* 22
3.1.1 *In silico* construct design 22
3.1.2 Cloning of the lacI-GFP fragment 22
3.1.3 Transformation into *V. cholerae* 25
3.2 Growth rates of planktonic *V. cholerae* cells 27
3.3 Mutation frequency in planktonic cultures 30
3.3.1 Determination of the mutation frequency using the kan-lacI-GFP construct 30
3.3.2 Determination of the mutation frequency through spontaneous antibiotic resistance 32
3.4 Mutation frequency in biofilm cultures 33
3.4.1 Biofilm growth rate 33
3.4.2 Determination of the mutation frequency using the kan-lacI-GFP construct 34
3.4.3 CLSM images of biofilm cultures 35
4 Discussion 38
4.1 Natural transformation 38
4.2 Mutation frequency in planktonic cultures 38
4.3 Mutation frequency in biofilm cultures 40
4.4 Comparison 41
5 References 43

List of abbreviations

AmpR	Ampicillin resistant
bp	Basepair(s)
CLSM	Confocal laser scanning microscope
CmR	Chloramphenicol resistant
DASW	Defined artificial seawater
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substance
et al	Et alteri
g	Gram
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_{dest}	Distilled water
i.e.	Id est
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase(pairs)
kan	Kanamycin
KanR	Kanamycin resistant
L	Liter
LB	Luria-Bertani
µ	micro-
M	Mol per liter, molar
M9 MM	M9 minimal medium
min	Minute(s)
ml	Milliliter(s)
n	nano-
nm	Nanometer
ODx	Optical density at the wavelength x
ON	Over night
PCR	Polymerase chain reaction
pH	negative common logarithm of the concentration of protons
RifR	Rifampicin resistant
rpm	Revolutions per minute
s	Second(s)
SEM	Simple and efficient method

SOB	Super optimal broth
SOC	Super optimal broth supplemented with glucose
TAE	Tris-Acetate-EDTA
Tcc	total cell count
Tn	Transposon
Tris	Trishydroxymethylaminomethane
UV	Ultraviolet
V	Volt
wt	Wild type
x g	x gravitational acceleration
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
x% (w/v)	x weight percentage per volume
x% (v/v)	x volume percentage per volume

1 Introduction

1.1 Microbial biofilms

In most natural, clinical and industrial settings, microorganisms often exist in heterogeneous, spatially structured environments as sessile communities as opposed to free-living planktonic cells. These communities, referred to as biofilms, develop structures that are morphologically and physiologically differentiated from single cell bacteria (Davies et al., 1993; Davies and Geesey, 1995; O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Prigent-Combaret, 1999). A biofilm is defined as a microbial community that is attached to a biological or non-biological surface. Microorganisms account for about 20% of the biofilm, the extracellular matrix consisting of secreted proteins, polysaccharides, DNA and water for about 80%. The matrix serves the microorganisms as a mechanic protection and plays an important role in adhesion of the cells to each other as well as to the substrate surface.

Biofilm formation occurs in a multistep development: Microorganisms approach the surface, attach and then become immobilized on the surface. Microcolonies arise when cells move along the surface and associate with one another. Finally, a pronounced three-dimensional structure is formed. Disintegration of the biofilm can occur due to mechanical forces acting on the biofilm or as a consequence of a conditionally-induced change of behavior in one or more subpopulations within the biofilm community. It leads to an apparently coordinated removal of a large number of cells from the biofilm (Hansen and Molin, 2004). All of these processes are regulated by environmental factors such as nutrient availability, oxygen, temperature, osmolarity, pH, iron availability, etc. (O'Toole et al. 2000).

Classical microbiological cultivations methods such as pure cultures in liquid media or on agar plates, which are essential for initial characterization and cultivation of a bacterium, inadequately reflect the natural life style of most microorganisms. In nature prokaryotes predominantly live in surface associated multi-species communities. In aquatic habitats with low nutrient availability, microorganisms tend to colonize surfaces as the nutrient content at the surface is much higher than in open salt- or fresh water (Zobell, 1943): On the one hand nutrients are concentrated through adsorption to the surface; on the other hand the diffusion rate of exoenzymes and hydrolysates is lower. Surfaces also provide a degree of stability in the growth environment and might have catalytic functions through localizing cells in close proximity. Biofilm formation affords protection from a wide range of environmental challenges, such as UV exposure (Espeland and Wetzel, 2001), metal toxicity (Teitzel and Parsek, 2003), acid exposure (McNeill and Hamilton, 2003),

dehydration and salinity (Le Magrex-Debar et al., 2000), phagocytosis (Leid et al., 2002), and several antibiotics and antimicrobial agents (Stewart and Costerton, 2001; Mah and O'Toole, 2001). Substrates for biofilms can be biotic (animal and plant tissue) as well as abiotic (minerals, metals, plastic). The microbial surface colonization has crucial consequences; minerals can be dissolved due to the biological activity of the colonizing organisms, metals corroded (Beech and Sunner, 2004) and biological tissue infected and damaged (Fux et al., 2005). However, biofilm formation can also be exploited in several applications, such as waste water treatment and bioremediation (Nicolella et al., 2000; Singh et al., 2006). Due to this wide range of implications it is important to understand the regulation of biofilm adhesion and stability, as well as the resistance of biofilm cells to antibiotics, UV radiation and detergents. Advances in biofilm research might allow systematic control of these processes. Some intensively studied microorganisms that are capable of forming biofilms are *E. coli* K-12 (Reisner et al., 2003), *Staphylococcus epidermidis* (O'Gara and Humphreys, 2001), *Pseudomonas aeruginosa* (Klausen et al., 2006), *Vibrio cholerae* (Watnick and Kolter, 1999), *Salmonella typhimurium* (Ngwai et al., 2006) and *Shewanella oneidensis* MR-1 (Thormann et al., 2006).

1.2 Vibrio cholerae

For the experiments in this comparison study the curved-rod shaped gram negative bacteria *Vibrio cholerae* was the model organism of choice. *V. cholerae* is the causative agent of the endemic cholera, an infectious disease characterized by infection of the small bowel and the production of liquid stools. It is one of the most rapidly fatal illnesses leading to hypotension within an hour of the onset of symptoms in a previously healthy person; an infected patient can die within three hours without medical treatment through the loss of large amounts of liquid via rice watery stools, triggered by binding of the cholera toxin to the epithelial cells of the small intestine (Ryan, 2004). Seven pandemics by two *V. cholerae* biotypes have been recorded; the classical biotype caused the first six pandemics whereas *V. cholerae* El Tor, today's dominant biotype worldwide, is responsible for the seventh, still ongoing pandemic of cholera (Colwell, 1996).

V. cholerae is an opportunistic pathogen that can proliferate efficiently in areas of high substrate availability and naturally occurs in a variety of marine waters throughout the world (Morris, 2003). Its natural life cycle involves biofilm formation, persistence and detachment when the cells transition between different environmental surfaces such as the chitinous shell of *Crustaceae* and the epithelial layer of the human intestine (Huq et al., 1983; Colwell, 1996; Butler and Camili 2005). However, the bacteria can also persist as free-living cells in aquatic reservoirs and survive long term starvation conditions by entering a spore-like viable, but nonculturable stage (Xu et al., 1982).

V. cholerae is an excellent model organism for biofilm research as its chromosome has been sequenced (Heidelberg et al., 2000) and extensive studies on regulation of biofilm adhesion and stability have been conducted (Watnick and Kolter, 1999). This has led to recent advances at a molecular level in the biofilm field (Beyhan et al., 2007; Liu et al. 2007; Lim et al., 2007; Mueller et al., 2007; Müller et al., 2007).

In general, the overall succession in biofilm development in *V. cholerae* corresponds to the stepwise differentiation process described by Hansen and Molin in 2004. Formation of a heterogeneous three-dimensional *V. cholerae* biofilm occurs within 60h when cells are cultivated under laboratory conditions in minimal medium on a borosilicate glass surface in hydrodynamic flow chambers (Figure 1; Müller, unpublished data). It takes 48h until the sides of the flow channel surface are covered by a 10-20 μm thick, flat biofilm whereas the central section contains patchy areas with microcolonies, also referred to as mushrooms. After this timepoint the biofilm goes through its first detachment phase: the 60-80 μm thick spherical structures begin to dissolve from the inside out. Bright field microscopy shows that the interior of the dissolving structures consist of highly motile cells. Within 24h most of the mushrooms have hollowed out, leaving an outer ~10 μm thick shell of cells behind. After another 24h, i.e. at 96h post-inoculation, the shell of the empty spherical structures has dissolved as well, resulting in a mostly uncovered substratum surface. After this detachment event, a more homogeneous and flatter biofilm redeveloped. Biofilms older than 60h consist of a phenotypically diverse population with colony variants arising at high frequency (Müller, unpublished data).

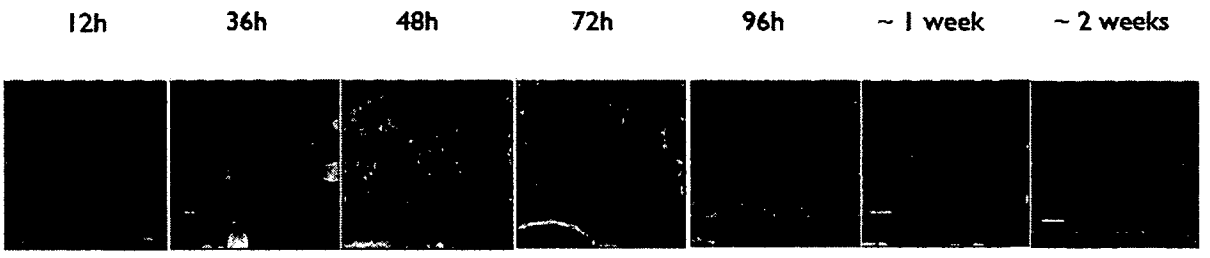

Figure 1. CLSM images of gfp-labeled *V. cholerae* A1552 smooth biofilms grown in a hydrodynamic flow chamber in a glycerol minimal medium at 30°C. Scale bars are 20 μm for 12h to 96h biofilms and 100 μm for 1 week and 2 week biofilms (Müller, unpublished data).

1.3 Evolution

Observations similar to those in *V. cholerae* described above have been made in other mono- and mixed species biofilms, respectively. Studies investigating phenotypic diversity have been conducted in a variety of biofilm forming microorganisms including *Staphylococcus epidermis* (Handke et al., 2004), *Pseudomonas aeruginosa* (Kirisits et al., 2005), *Listeria monocytogenes* (Monke et al., 2004), *Serratia marcescens* (Kohe et al., 2007) and *Pseudomonas fluorescens* (Rainey and Travisano, 1998). It has been previously shown that biofilm-associated organisms are able to adapt to environmental changes by altering their gene expression and general physiology (Davies et al., 1993; Davies and Geesey, 1995; OToole and Kolter, 1998; Pratt and Kolter, 1998; Prigent-Combaret et al., 1999), including increased resistance to antibiotics (Anwar et al., 1992; Brooun et al., 2000; Costerton et al., 1999). However, the genetic contribution to the increased phenotypic variation in biofilms has not been accounted for. On a genetic level, diversity can be created by the evolutionary forces of mutation, recombination, migration, drift and natural selection (Rainey et al., 2000).

Mutation is defined as "the process by which a DNA base-pair change or chromosome change is produced. A mutation, then, is the DNA base-pair change or chromosome change resulting from the mutation process" (Russell, 1998). This process can occur spontaneously or be induced by mutagens. Mutations can be distinguished into four categories regarding the base pairs that are affected. Hence, a point mutation is a single base pair change that can be caused either by the chemical conversion of one base into another or by mistakes that occur during replication.

A transition is a replacement of a G-C base pair with an A-T base pair or vice versa. A transversion replaces a purine with a pyrimidine, such as changing A-T to T-A. The most common type of mutation is an insertion, which results from the movement of transposable elements. The effects of these mutations can be reversed by back mutations, deletions of inserted material or by suppression, i.e. through a mutation in a second locus bypassing the effect of the mutation in the first locus. The mutation frequency at any particular base pair is determined by statistical fluctuation. However, at so-called hotspots within the genome, mutations accumulate leading to a frequency that is increased by at least an order of magnitude (Lewin, 2007).

1.4 Goals

In this work the general question whether the phenotypic variation observed in *V. cholerae* is due to genotypic differences was to be elucidated by focusing on one aspect of mutagenesis, the spontaneous mutation of cells. The working hypothesis was that the

molecular basis for phenotypic variations is random mutation. The goal was to study the underlying molecular mechanisms that lead to phenotypic diversity. Moreover, the phenotypic variation observed in biofilms was to be quantified by measuring mutation frequencies. This would enable a comparison of the biofilm mutation frequencies to those determined for planktonic cell populations.

In order to measure mutation frequencies, a genetic tool was to be used as a "mutation trap"; the construct, which was to be introduced into *V. cholerae*'s chromosome via natural transformation upon induction of natural competence by growth on chitin, carried a *gfp* and a kanamycin resistance gene both under the control of the P_{lac} promoter. In this system, the lac promoter will be repressed by the lac product, a tetramer binding to the operator region. If either the *lacI* itself or the operator region acquire a mutation that prevents binding of the repressor, the expression of *gfp* and the kanamycin resistance gene would increase. The premise was that cells would thus fluoresce and be kanamycin resistant, which would make it possible to determine the ratio of cells that acquired a mutation, i.e. the relative mutation frequency, by plating onto agar plates with and without kanamycin. Simultaneously, the goal was to monitor biofilm cells for GFP expression by Confocal Laser Scanning Microscopy (CLSM), thereby enabling the observation of evolution in real-time in the biofilm. In a parallel approach, mutation frequencies were to be estimated by measuring the spontaneous acquisition of antibiotic resistance to streptomycin, spectinomycin or nalidixic acid.

2 Material and methods

2.1 Bacterial strains, plasmids and oligonucleotides

The *V. cholerae* strains used were smooth phase variants of A1552 El Tor, Inaba, wild type, RifR (Yildiz and Schoolnik, 1998) and hapR complemented N16961 (Nielsen et al., 2006). The *E. coli* strain DH5α-λpir was used for standard DNA manipulation experiments (Miller and Mekalanos, 1988), and the *E. coli* strain S17–1-λpir was used for conjugation with *V. cholerae* (Simon et al., 1983).

The plasmids and oligonucleotides used in this study are listed in tables 1 and 2.

Table 1. Plasmids

Plasmid		Reference
pGP704	Derivative of pJM703.1; oriR6K, mobRP4, AmpR	Miller and Mekalanos, 1988
pGP704::lacIQ-GFP-Cm	Derivative of pGP704; GFP and CmR controlled by P_{lac}	Plasmid pSS41, Spormann AM, unpublished
pBR322::lacZ-kan-SacI-lacZ	Derivative of pBR322; promoterless kan resistance cassette in the lacZ gene	Blokesch M, unpublished
pBR322::lacZ-kan-GFP-lacI-lacZ	Derivative of pBR322::lacZ-kan-SacI-lacZ; GFP and kanR controlled by P_{lac}	This study

Table 2. Oligonucleotides

MF F1 lacZ SacI	GCGGAGCTCAGCTAATTAAGCTTATTTGTATAGTTCATCC
MF R1 lacZ SacI	AATGCGGAGCTCACTGCCCGCTTTCCAG
kanRStartbw	GCAAGACGTTTCCCGTTGAATATGGC
AB GFP seq #1	AGTTCTTCTCCTTTACGCATGC
AB lacZ seq #1	GCTAAATCCAGCGTGATTGTGCC

Oligonucleotides were synthezised by PAN Oligo Synthesis Service (Stanford, California, United States), delivered in lyophilized form, dissolved in H_2O_{dest} to a final concentration of 100 μM and stored at -20°C.

2.2 Media

Bacteria were grown either in standard LB broth with 0.5% NaCl (Becton, Dickinson & Company, Sparks, Maryland, USA) or in M9 Minimal medium (M9 MM), supplemented

with the carbon source glycerol to a final concentration of either 50mM for planktonic cultures or 1.5 mM for biofilm cultures (Sambrook et al., 2001).

SOB medium (20 g Tryptone, 6 g Yeast extract, 10 mM NaCl, 2.5 M KCl, 10 mM $MgCl_2$ x 6 H_2O, 10 mM $MgSO_4$ x 7 H_2O in 1l H_2O_{dest}) and SOC medium (SOB plus glucose) were prepared as described by Hanahan, 1983. Terrific Broth (TB; 10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, $MnCl_2$, pH 6.7) was prepared following the protocol by Inoue et al., 1990.

Defined artificial seawater (DASW) was prepared as follows: 250 ml DASW stock, 2x 125 ml Hepes 100 mM, pH 7.4 and 5 ml MEM Vitamin Solution 100x (GIBCO) were mixed with 125 ml $H2O_{dest}$. The solution was filter sterilized, stored at RT and used within a week.

If plates were to be poured of a certain nutrient solution, 1.5 % (w/v) Bacto-Agar (Becton, Dickinson & Company) was added to the media for solidification.

If not specified otherwise antibiotics and other supplements were added to the following final concentrations:
Ampicillin: 100 µg/ml
Rifampicin: 100 µg/ml
Isopropyl-ß-D-thiogalactopyranosid (IPTG): 0.5 mM
Kanamycin: 75 µg/ml (kan75) and 150 µg/ml (kan150)
Vitamins: 1x MEM vitamin solution 100× (GIBCO)
5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal): 80µg/ml

2.3 Growth conditions

2.3.1 Planktonic *E. coli* cultures

E. coli strains were grown at 37°C in LB medium containing the adequate antibiotic. Culture volumes up to 5 ml were grown in 15 ml tubes on a roller, bigger volumes were incubated in suitable sized Erlenmeyer flasks with vigorous shaking. Freezer stocks of the cultures were made using 400 µl of ON LB cultures mixed with 600 µl of 50% sterile glycerol and stored at -80°C.

2.3.2 Planktonic *V. cholerae* cultures

Frozen stock *V. cholerae* cells were thawed, streaked on an LB agar plate and incubated ON at 30°C. Plates were never kept longer than 2 days, because of *V. cholerae*'s high mutation rate. A colony was picked with a plastic loop, inoculated in LB medium or 50mM glycerol M9 MM containing the appropriate antibiotic if needed, and grown on a roller at 30°C either ON or until a certain desired OD_{600} was reached. Larger culture volumes were grown in suitable sized Erlenmeyer flasks with vigorous shaking. Freezer stocks of the cultures were made as described for *E. coli*.

2.3.3 Preparation of competent *E.coli* DH5α-λpir cells

E. coli DH5α-λpir cells were made competent using the simple and efficient method (SEM) by Inoue H. et al. (1990) which is a slight modification of the protocol described by Hanahan (1983):
Frozen lab stock DH5α-λpir cells were thawed, streaked on an LB agar plate and incubated ON at 37°C. A dozen colonies were picked with a plastic loop, inoculated to 250 ml of SOB medium in a 2-liter flask, and grown to an OD_{600} of 0.6 at 18°C and 250 rpm. All the following steps were performed on ice and at 4°C, respectively, if the step required centrifugation. The flask was removed from the incubator. After 10 min. the culture was transferred to a 500 ml centrifuge bottle and spun at 2500 x g for 10 min. The supernatant was discarded, the cell pellet gently resuspended in 80 ml of ice-cold TB, incubated for 10 min. and again centrifuged as described above. The cell pellet was resuspended in 20 ml of TB and dimethylsulfoxide (DMSO) was added with gentle swirling to a final concentration of 7%, i.e. 1.5 ml DMSO to a total volume of 21.5 ml. The cell suspension was incubated for another 10 min. and then dispensed as 200 µl aliquots into 1.5 ml centrifuge tubes and immediately immersed in liquid nitrogen. The frozen competent cells were stored at -80°C.

2.4 Strain constructions in *V. cholerae*

In brief, the lacI and GFP genes were amplified by PCR using the vector pGP70::lacI-GFP-Cm, introducing suitable restriction sites. The product was purified, digested with SacI, and ligated into a SacI digested pBR322 derived vector containing a promoterless gene coding for kanamycin resistance introduced into the *V. cholerae* lacZ gene, kindly provided by Dr. Melanie Blokesch. The resulting plasmid (pBR322::lacZ-kan-GFP-lacI-lacZ) was transformed into chemically competent *E. coli* cells, and transformants were selected on LB plates containing kanamycin and IPTG. Colonies were then screened for the *lacI-GFP* insertion twofold: 1) by PCR using primers specific for the *lacI* gene and the

lacZ gene, respectively, which ensured the correct orientation of the fragment relative to the kan cassette. 2) by SacI restriction digest of the purified plasmid. Plasmid preps of the positive clones were sent for sequencing. The plasmid was digested with NheI, and the linear DNA fragment was introduced into the *V. cholerae* genome by natural transformation. Positive clones were then screened 1) by plating on LB-Agar plates containing the antibiotic kanamycin and IPTG, 2) by blue white screening on LB-Agar plates supplemented with X-Gal, and 3) by colony PCR optimized for *V. cholerae*.

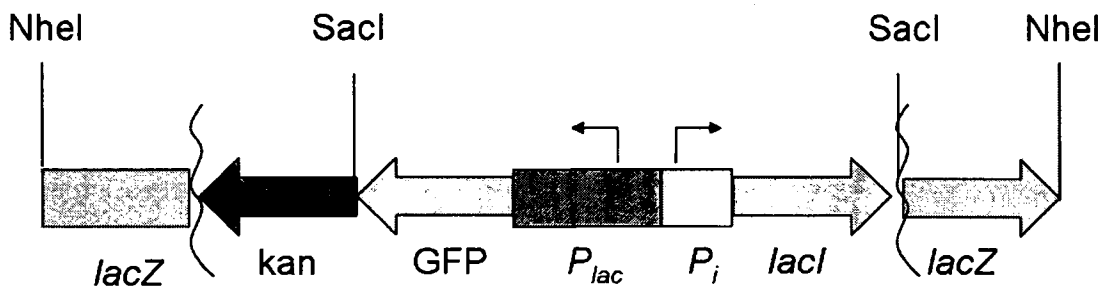

Figure 2. Schematic depiction of the final lacZ-kan-GFP-lacI-lacZ construct.

2.4.1 *In silico* design of cloning experiments

Prior to the actual experimental work, the final lacZ-kan-GFP-lacI-lacZ construct was created *in silico* using Vector NTI Advance 10.3.0 (Invitrogen, Carlsbad, CA, USA) for sequence creation, mapping, analysis, annotation and illustration. This software was also used for alignment of sequencing data.

2.4.2 DNA Amplification by Polymerase Chain Reaction (PCR)

Using the PTC-200 Peltier Thermal Cycler (MJ Research, Ramsey, MN, USA) and specific primers fragments encoding the lacI and GFP genes were amplified out of the vector pGP70::lacI-GFP-Cm by PCR. In all reactions the enzyme PfuUltra Hotstart DNA polymerase (Stratagene, La Jolla, CA, USA) and its corresponding 10x PfuUltra HF Reaction Buffer were used to ensure high accuracy and robust amplification of long targets. The sequences of the primers used are listed in Table 2.

PCR setup

Component	per reaction
Template DNA (100ng/µl)	1 µl
10x PfuUltra HF Reaction Buffer	5 µl
dNTPs 10 mM	1 µl
Forward primer (100 µM)	0.25 µl
Reverse primer (100 µM)	0.25 µl
PfuUltra Hotstart polymerase (2.5 U/µl)	1 µl
Nuclease free water	41.5 µl
Total reaction volume	50 µl

8 identical reactions were set up by preparing a master mix and aliquoting it à 50 µl in 200 µl thin wall tubes, and run in a PCR with a temperature gradient to determine the optimal annealing temperature. The course of a typical program used in the lab would be:

1)	95 °C	5 min	
2)	95 °C	30 sec	} 25 cycles
3)	55 – 68 °C	30 sec	} 25 cycles
4)	72 °C	2 min 30 sec	
5)	72 °C	10 min	

The individual annealing temperatures resulting from the temperature cycling gradient in step three ranged from 58.7°C to 68°C. The extension time of 2 min 30 sec in step four corresponds to 1 min per 1 kb.

If the yield of this first reaction was not satisfactory the PCR was repeated, using the optimal annealing temperature and the product of the first reaction as a template for the second.

The synthesized fragments were kept either at 4°C or -20°C for long term storage.

2.4.3 Agarose gel electrophoresis

DNA fragments were separated and purified by preparative agarose gel electrophoresis. Therefore 1% (w/v) agarose in TAE buffer (40 mM Tris-Acetate, pH 8.0, 1 mM EDTA) was boiled in the microwave, supplemented with ethidium bromide to a final concentration of 0.5 µg/ml before pouring, and left to solidify in the electrophoresis chamber. Samples were mixed with loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylenocyanol, 15% (w/v) ficoll 400; Sambrook und Russell, 2001) and pipetted into the sample wells. The 1 Kb Plus DNA Ladder (1 µg/µl in 10 mM Tris HCl, pH 7.5, 50 mM

NaCl, 1.0 mM EDTA; Invitrogen) was used for qualitative and quantitative DNA estimation. The running buffer was TAE buffer as well. Separation was carried out at 80 V (constant voltage), the running time depending on fragment length. The separated bands were visualized by UV-transillumination at 365 nm.

2.4.4 DNA extraction from agarose gels

The extraction of DNA from agarose gels was performed using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The DNA was eluted in elution buffer diluted 1:4 with H_2O. Success of the extraction (fragment length, purity and amount of DNA) was analyzed electrophoretically. The DNA concentration was also determined via ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Wilmington, DE, USA), a full-spectrum UV/Vis spectrophotometer used to quantify nucleic acids, proteins, fluorescent dyes and other compounds.

2.4.5 Purification of DNA from solution

PCR products and other DNA fragments in solution were purified using the QIAquick PCR purification Kit (Qiagen), eluted in 30 µl of elution buffer diluted 1:4 with H_2O. The analysis of fragment length and purity as well as the determination of the DNA concentration were performed as described for DNA extracted from agarose gels.

2.4.6 Enzymatic Restriction

Sequence-specific DNA restriction was carried out using enzymes from New England Biolabs (NEB; Beverly, MA, USA). Digest conditions and enzyme concentrations were individually adjusted according to the manufacturer's directions.

Thus, 20 units, i.e. 1µl, of the enzyme SacI were used per µg of purified lacI-GFP insert and pBR322::lacZ-kan-lacZ vector DNA, respectively, in an enzymatic reaction buffered with NEBuffer 1 (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.0 at 25°C) and supplemented with 100 µg/ml Bovine Serum Albumin (BSA), that took 1h at 37°C.

10 units, i.e. 1 µl, of the restriction enzyme NheI were used to linearize 1 µg of the final plasmid pBR322::lacZ-kan-GFP-lacI-lacZ in the presence of NEBuffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9 at 25°C) and 100 µg/ml BSA in 1h at 37°C.

The restricted fragments were analyzed and purified by agarose gel electrophoresis followed by gel extraction, and the DNA concentration was determined by ND-1000 Spectrophotometer (NanoDrop).

2.4.7 Vector dephosporylation

To reduce vector background in the succeeding cloning steps Antarctic Phosphatase (NEB) was used for dephosphorylation of the digested vector following the manufacturer's protocol; that is 5 units, i.e. 1µl, of the enzyme were used per µg of digested vector DNA to catalyze the removal of 5' phosphate groups in 1h at 37°C in an enzymatic reaction buffered with Antarctic Phosphatase Reaction Buffer (50 mM Bis-Tris-Propane-HCl, 1 mM $MgCl_2$, 0.1 mM Zn Cl_2, pH 6.0 at 25°C). After Antarctic Phosphatase treatment, the DNA was purified using the QIAquick PCR purification Kit (Qiagen) as described above.

2.4.8 Ligation

The SacI digested lacI-GFP fragment was ligated into the dephosphorylated vector pBR322::lacZ-kan-lacZ in a vector : insert molar ratio of either 1:1 or 1:3, using the Quick Ligation Kit (NEB). According to the manufacturer's protocol ligation with recombinant Quick T4 DNA Ligase was carried out in the presence of Quick Ligation Buffer (66 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 7.5 % Polyethylene glycol (PEG 6000), pH 7.6 at 25°C) in 5 minutes at RT. As a negative control, in order to estimate the degree of self-ligation, one ligation reaction without insert DNA was set up.

2.4.9 Transformation of chemically competent *E. coli* cells with plasmids

Chemically competent lab stock DH5α cells were transformed following the protocol of Dr. Kai Thormann. The entire ligation product was thereby mixed with 200 µl of competent cells and left on ice for 30 - 45 min. The tube was heat shocked in a 42°C water bath for exactly 1 min and cooled on ice for another 2 min. 800 µl of SOC medium were added to the tube. To recover the transformed cells were incubated on a roller for 1 h at 37°C. The culture was spun down briefly and the cell pellet resuspended in 100 µl LB. 100 µl of this concentrated cell suspension as well as 100 µl of the original culture were spread on LB plates containing a selective antibiotic and incubated at 37°C.

E. coli One Shot® Mach™1 Chemically competent cells (Invitrogen) were transformed with 10 µl ligation product according to the protocol provided by the manufacturer and the

transformed cells were plated on LB-agar plates containing 75 µg/ml of the selective antibiotic kanamycin as well as IPTG to induce resistance.

Transformation efficiency and success were determined both by colony PCR using primers specific for the *lacI* and the *lacZ* gene, so as to check whether the orientation of the fragment relative to the kan cassette was correct, and by SacI restriction digest of the purified plasmid (see 2.4.6).

2.4.10 Colony PCR

PCR was used to screen for positive transformants. Therefore ON colonies were selected with a tooth pick and transferred into the Go Taq Green Master Mix (Promega, Madison, WI, USA) supplemented with lacI and lacZ specific primers listed in Table 2 in 200 µl thin wall tubes. The reaction set up is listed in the following table:

PCR setup

Component	per reaction
Go Taq Green Master Mix	12.5 µl
Forward primer (100 µM)	0.2 µl
Reverse primer (100 µM)	0.2 µl
Nuclease free water	12.1 µl
+ DNA from picked colony	-
Total reaction volume	25 µl

The same instrument and temperature cycling parameters were used as described in 2.4.2 with the exceptions that the annealing temperature was 50°C and the extension time 1 min 30 sec which corresponds to 1 min per kb.

The picked colonies were also spread on LB-agar plates containing the appropriate antibiotic for further cultivation of positive clones.

2.4.11 Freezer stocks

Clones that proved to contain the desired plasmid were cultivated, and freezer stocks of them were made as described in 2.3.1.

2.4.12 Plasmid purification

The isolation of plasmid DNA from transformed *E. coli* cells was performed using the QIAprep Miniprep Spin Kit (Qiagen), or if bigger quantities of DNA were required, the QIAGEN Plasmid Midi Kit (Qiagen) following the manufacturer's protocol. In both cases

the DNA was eluted and in 50 µl prewarmed elution buffer diluted 1:4 with H_2O. Success of the isolation (purity and amount of DNA) was analyzed electrophoretically. The DNA concentration was also determined via ND-1000 Spectrophotometer (NanoDrop). Isolated plasmid DNA was stored at -20°C.

2.4.13 Sequencing and sequence analysis

Purified plasmid DNA of the positive clones was sent to the MCLab (South San Francisco, CA, USA) for sequencing, and the obtained sequence data was processed using Contig Express, Vector NTI Advance 10.3.0 (Invitrogen).

2.4.14 Natural transformation on crab shell surfaces

The transformation of *V. cholerae* strains was performed as described in the protocol by Meibom et al. (2005); frozen stock *V. cholerae* cells were thawed, streaked on an LB-agar plate and incubated ON at 30°C. A colony was picked with a plastic loop, inoculated to 6 ml of LB medium, and grown ON at 30°C on a roller. 100 µl of the culture were transferred into 6 ml of fresh LB medium and grown to an OD_{600} of 0.3 under the same conditions. The tube was removed from the incubator and the culture was transferred to a centrifuge tube and spun at 6000 x g for 10 min at 30°C. The supernatant was discarded, and the cell pellet washed with DASW by first resuspending the pellet in 1 ml of the solution and then adding another 5 ml. The second centrifugation step was performed as described above. The cell pellet was resuspended in a total volume of 6 ml as described above and dispensed by 1 ml into 6 wells of a 12 well plate, containing a piece of autoclaved crab shell each as a chitin source.

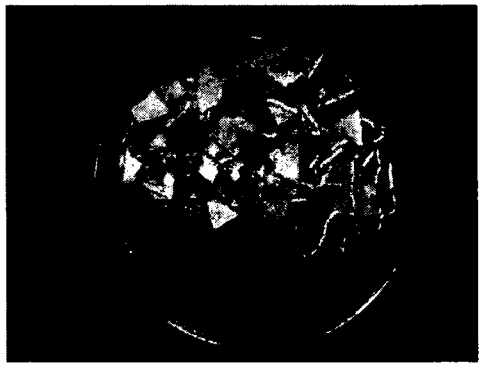

Figure 3. Autoclaved crab shells as a chitin source in the natural transformation experiment.

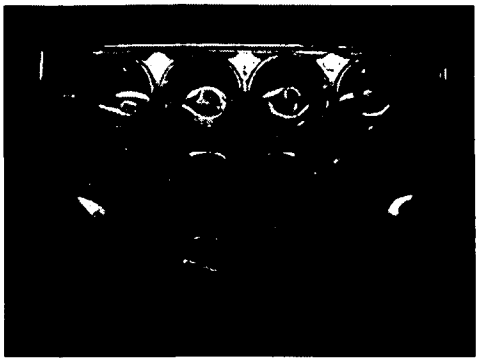

Figure 4. 12-well plate containing crab shells, media and *V. cholerae* cells.

The cultures were incubated ON at 30°C. The following day the media was replaced with 2 ml of fresh DASW. Approximately 2 µg of DNA were added to each well. Then the plate was incubated at 30°C for another 24 h in which transformation would take place. The cells were washed with DASW by sterilely dipping the crab shells into fresh DASW. The crab shell was then put in a 50 ml tube containing 1 ml of DASW and vortexed for 30 sec. The following dilution series was prepared for plating on the respective plates:

Dilution	Plate
10E0	LB+kan75+IPTG
10E-2	LB+kan75+IPTG
10E-4	LB+X-gal
10E-6	LB, LB+X-gal

100 µl from each dilution step were spread on the respective plate in triplicates, and the plates were incubated ON at 30°C.

Positive clones would then either grow on LB-Agar plates containing the antibiotic kanamycin and IPTG or appear white on LB-Agar plates supplemented with X-Gal.

2.4.15 *V. cholerae* colony PCR

PCR was used to screen for *V. cholerae* transformants containing the kan-GFP-lacI construct in the lacZ gene. Therefore an ON colony was suspended in 100 µl of $H_2O_{dest.}$ and 10 µl of the suspension were transferred into a 200 µl thin wall tube. In the PTC-200 Peltier Thermal Cycler (MJ Research) the solution was kept at 95°C for 15 min and after another 10 min at 80°C Go Taq Green Master Mix (Promega) supplemented with GFP specific primers (listed in Table 2) was added directly to each tube in the thermal cycler to a final reaction volume of 25 µl.

The following PCR temperature cycling parameters were used:

1)	94 °C	30 sec	30 cycles
2)	55 °C	30 sec	
4)	72 °C	60 sec	
5)	72 °C	10 min	

The picked colonies were also spread on LB-agar plates containing the appropriate antibiotic and supplement if necessary, for further cultivation, and freezer stocks of clones containing the kan-GFP-lacI construct in the lacZ gene were made as described in 2.3.2.

2.5 Experiments with planktonic cultures of *V.cholerae*

Cultivation of planktonic *V. cholerae* cultures in growth curve, mutation frequency and antibiotic susceptibility experiments was essentially carried out as described in 2.3.2.

2.5.1 Growth curves

ON LB cultures of the respective *V. cholerae* strains were diluted to an OD_{600} of 0.05 in 50 ml LB and 25 ml 50 mM glycerol M9 MM, respectively, and grown according to 2.3.2.
Samples of the LB cultures were taken every 45 min for 3h, thereafter every 90 min until stationary phase at 7.5 h postinoculation. M9 MM cultures were sampled every three hours until 30h postinoculation.

2.5.2 Mutation frequency determination

ON LB cultures of the respective *V. cholerae* strains cointaining the kan-lacI-GFP construct in lacZ were diluted to an OD_{600} of 0.05 in 50 ml LB and 25 ml 50 mM glycerol M9 MM, respectively, and grown as described in 2.3.2.
Samples of the mid-log (OD_{600} ~ 0.4), late-log (OD_{600} ~ 1), stationary phase (OD_{600} ~ 5) and 1 week old LB cultures were drawn, diluted accordingly and plated on both plain LB plates for the total cell count determination and on LB plates containing kan150 for the mutated cell count determination.
50 mM glycerol M9 MM cultures were sampled, appropriately diluted and plated on plain LB plates and LB plates containing kan150 in log (OD_{600} ~ 0.4) and stationary phase (OD_{600} ~ 2), i.e. 6h and 24h postinoculation.
Subsequently the mutation frequency was calculated by dividing the mutated cells by the total cell count, and the data were displayed in a bar chart in Microsoft Office Excel 2003 SP2.

2.5.3 Determination of acquisition of antibiotic resistance through point mutations

ON LB cultures of wild type *V. cholerae* strains were diluted to an OD_{600} of 0.05 in 5 ml fresh LB medium and grown to mid-log phase as described in 2.3.2. The cultures were diluted accordingly and plated on both plain LB plates for the total cell count determination and on LB plates containing 25, 50 and 100 µg/ml streptomycin or spectinomycin and 5, 10 and 25 µg/ml nalidixic acid, respectively, for the mutated cell count determination. LB plates were incubated ON, plates containing an antibiotic for 48h. The mutation frequency was calculated by dividing the number of cells counted on plates containing the respective antibiotic, i.e. the mutated cells, by the total cell count.

2.6 Experiments with *V.cholerae* biofilm cultures

V. cholerae biofilms were grown as described previously (Thormann et al., 2004). All biofilm characterizations were conducted in triplicate in at least two independent experiments.

2.6.1 Biofilm setup

Biofilm studies were conducted on a borosilicate surface in three-channel flow cells under hydrodynamic flow conditions; the individual channel dimensions were 1 by 4 by 40 mm. Each flow chamber was prepared as follows: a microscope coverslip (Fisher Scientific, Pittsburgh, PA, USA), which served as a substratum for microbial attachment, was glued onto the flow chamber with silicone (GE Sealants & Adhesives, Hunterville, NC, USA) and left to dry for 24 h at room temperature prior to use. Essentially, the flow system was assembled as described earlier (Christensen et al., 1999) with the exception that the setup was sterilized by autoclaving for 20 min before the inflow tubing was connected to the medium bottle. After assembly, the set up was equilibrated with medium at a low flow rate of 0.5 rpm for at least 6 h at 30°C before inoculation, using a Watson-Marlow Bredel 205S peristaltic pump (Cornwall, United Kingdom).

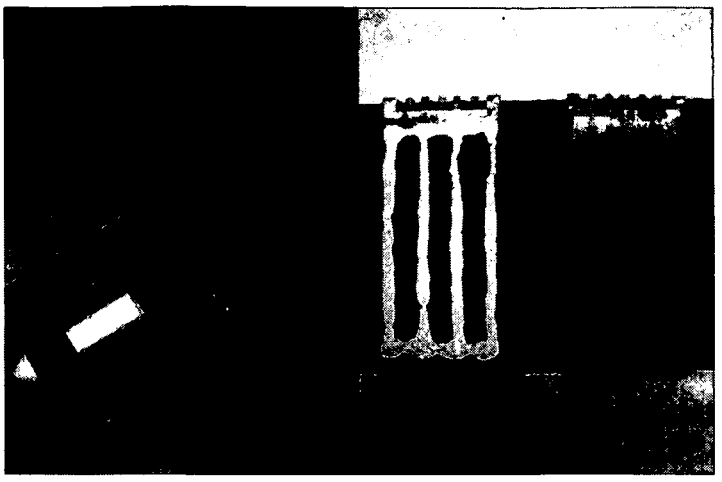

Figure 5. Flow chamber assembly: *a.* The microscope coverslip is glued on to the flow chamber with silicone. *b.* Borosilicate flow chamber without top on the right, with top on the left.

Figure 6 a. Biofilm setup: Media bottles, inflow tubing, peristaltic pump, bubble traps, three-channel flow cells, outflow tubing, connection to waste. *b.* Close-up of bubble traps.

2.6.2 *V. cholerae* biofilm cultures

ON cultures of the respective *V. cholerae* strain were grown as described in 2.3.2. The cultures were diluted 1:100, i.e. 50 µl in 5 ml of fresh LB supplemented with the appropriate antibiotic and grown to mid-logarithmic phase. Then the optical density at 600 nm was diluted to 0.01 in M9 MM without carbon source, i.e. glycerol.

All the following steps were performed at 30°C. After stopping the medium flow and clamping off the inflow tubing one inch above its connection to the flow channel, 1 ml of the adjusted cell suspension was injected into each flow channel with a syringe. The chambers were turned upside down to enhance initial attachment and incubated for 40 min. Then the flow cells were inverted, and the medium flow was started again at a constant velocity of 0.3mm/s per channel.

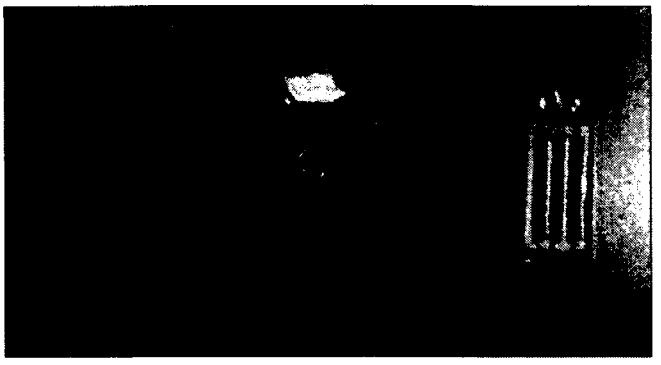

Figure 7a. Injection of *V. cholerae* cells into the flow channel. *b.* Flow cell with in- and outflow tubing after injection.

2.6.3 Harvesting biofilm cells

Biofilm cells were harvested at 48, 60 and 168 h postinoculation according to a protocol by Soni Shoukla: the in- and outflow tubing of the flow cell of interest were clamped, treated with 70 % ethanol and then cut off with a sterile razor blade. 500 µl of LB medium were injected into the flow channel and sucked up again using a syringe. This rinsing of the flow channel was repeated multiple times to ensure that all cells were washed out and homogeneously suspended in the medium and that no cell clumps remained.

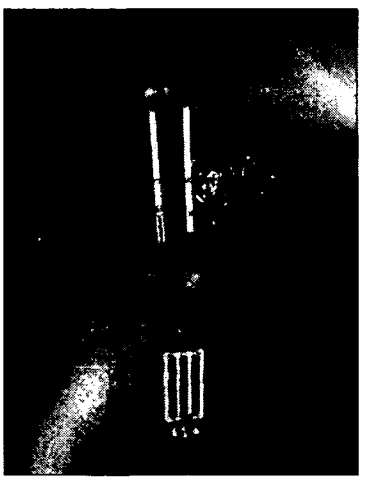

Figure 8. Harvesting the biofilm cells.

Dilution series of the biofilm cells were made ranging from 10E0 to 10E-6 depending on the time point of harvesting. Cells were plated on both plain LB plates for the total cell count determination and on LB plates containing kan150 for the mutated cell count determination.

Subsequently the mutation frequency of the biofilm cells was calculated and the data were displayed analogously to those for the cells in planktonic cultures (described in 2.5.2).

2.6.4 Cell staining with the SYTO 63 red fluorescent nucleic acid stain

The cell-permeant SYTO63 red fluorescent nucleic acid stain (Invitrogen) exhibits red fluorescence upon binding to nucleic acids. Thus, cell staining with this dye enables visualization of the total biofilm.

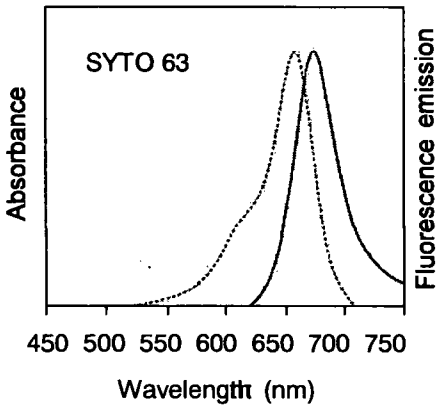

Figure 9. Normalized absorption and fluorescence emission spectra with SYTO 63 stain bound to DNA. Max. absorption: 654 nm, max. emission 675 nm. (Plot provided by Invitrogen, *Spectra for Red Fluorescent SYTO® Dyes.*)

Cells were stained according to the manufacturer's protocol: the Syto63 stock solution (5mM in DMSO) was diluted in M9 biofilm medium to a 5 μM final concentration, covered in foil and kept on ice until use.
With a syringe, the medium in the bubble trap was emptied out and the tubing was disconnected from the medium bottle supply to avoid dilution of the stain due to medium inflow. Then 2 ml Syto63 were added into the bubble trap with a syringe. It took about 10 min for the stain to reach the flow cell and another 15 min for the staining itself. Subsequently, the tubing was reconnected to the medium bottle and the flow cell was washed for 20 min with M9 biofilm medium. Images of the stained biofilm cultures were acquired and processed as described in 2.6.5

2.6.5 Image acquisition and processing

Biofilms were imaged at 48, 60 and 168 h postinoculation with an upright LEICA TCS SP2 AOBS confocal laser scanning microscope (CLSM; Leica Microsystems, Wetzlar, Germany) using the objectives HCX PL APO 63X/1.2 W CORR CS and HC7

PL FLUORTAR 20X/0.5. GFP was scanned with the Ar/ArKr laser at 488/550 nm, Syto63 with the He/Ne laser at 654/675 nm. The z-stacks were reconstructed into z-projections using the Imaris-software (Bitplane AG, Zürich, Switzerland) and figures were assembled with Photoshop CS (Adobe, San Jose, California, United States).

3 Results

3.1 Strain constructions in *V. cholerae*

Construction of *V. cholerae* strains carrying the kan-lacI-GFP mutation trap by the method using the Tn7-based delivery system (Lambertsen et al., 2004) previously conducted in the lab, was found to be technically difficult. (Only later it was discovered that not the method itself, but a faulty UV transilluminator used in several steps of the biomolecular procedure, was to blame for the lack of cloning success.) Therefore a novel strategy for construction of *V. cholerae* mutants was applied which takes advantage of the bacteria's natural competence upon chitin induction to acquire new genetic material by natural transformation (Meibom et al., 2005).

3.1.1 *In silico* construct design

Using Vector NTI Advance, SacI restriction sites were added to the lacI-GFP fragment of the lacZ-lacI-GFP-kan-lacZ construct *in silico*. An illustration of the fragment is shown in Figure 10. The sequence was annotated and virtually cloned into the pBR322::lacZ-kan-SacI-lacZ vector.

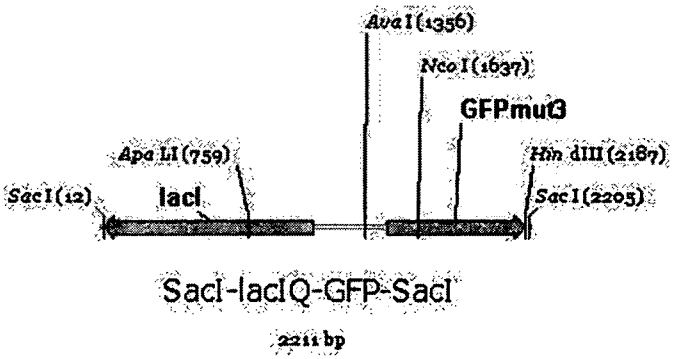

Figure 10. Schematic depiction of the SacI-GFP-lacI-SacI containing DNA fragment (VectorNTI).

3.1.2 Cloning of the lacI-GFP fragment

The region containing the lacI and GFP genes from the plasmid pGP704::lacI-GFP-Cm was PCR-amplified introducing flanking SacI restriction sites resulting in a ~2200 bp fragment (see Figure 11).

Figure 11. PCR products of the amplified SacI-lacI-GFP-SacI fragment. L indicates the 1kb DNA Plus Ladder, 1 to 3 represent the PCR products generated at different temperatures in the gradient cycler.

The purified PCR product was restricted with the enzyme SacI for further cloning and with NcoI to confirm the amplified fragment, resulting in bands at 570 bp and 1630 bp if correct. The digested DNA is shown in Figure 12.

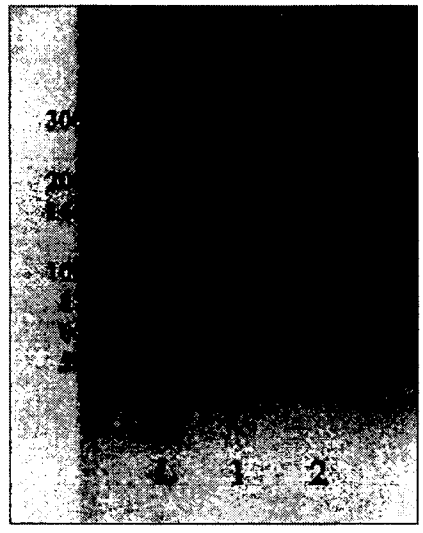

Figure 12. Digested SacI-lacI-GFP-SacI fragment. L indicates the 1kb DNA Plus Ladder. 1 represent the NcoI digested fragment with bands at 570 bp and 1630bp. 2 shows the SacI digested fragment at 2200 bp.

The thereby confirmed and SacI digested SacI-lacI-GFP-SacI fragment was cloned into the SacI restriction site of the digested and dephosphorylated plasmid pBR322::lacZ-kan-SacI-lacZ (kindly provided by Dr. Melanie Blokesch). No transformants were obtained using *E. coli* strains DH5α. The plasmid, however, could be transformed into One Shot® Mach™1 chemically competent *E. coli* cells though. Colonies grown on LB plates containing 75 μg/ml of the selective antibiotic kanamycin and 0.5mM IPTG showed two distinct sizes. This can be explained by the fact that depending on the orientation of insertion of the *lacI-GFP* fragment the gene conferring kanamycin resistance is either constitutively expressed - resulting in big colonies - or under lac repression and thus only expressed after binding of IPTG to the lac repressor, which will lead to smaller colonies.

12 small and 12 large colonies were picked and screened via colony PCR for successful *lacI-GFP* insertion, confirming the correct orientation of the fragment relative to the kan cassette. As expected, PCR of the large colonies was negative. The results of the colony PCR of the small colonies with primers specific for the *lacI* and *lacZ* gene are shown in Figure 13.

Figure 13. Transformants were screened in a colony PCR using *lacI* and *lacZ* specific primers, resulting in a ~1 kb fragment when transformation was successful. Numbers 1 to 12 indicate the picked colony, L the 1 Kb Plus DNA Ladder used as a reference.

Purified plasmid DNA of clones 3, 9 and 11 and five other randomly picked small colonies was digested with SacI, resulting in a second band at 2.2 kb (Figure 14).

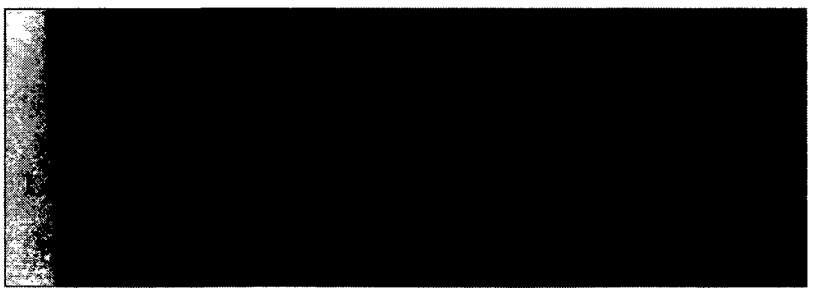

Figure 14. SacI digestion of purified plasmid DNA. L indicates the 1 Kb Plus DNA Ladder used as a reference, numbers specify the respective plasmid DNA. Bands of plasmids 3 and 9 are shown both uncut and cut. Digested plasmids are expected to show a band at 2.2 kb if positive.

Plasmid DNA of the positive clones 1, 3 and 9 was sent for sequencing to McLab. The sequence of the final lacZ-kan-GFP-lacI-lacZ construct was confirmed by alignment of the sequencing data with the virtual construct using ContigExpress from the VectorNTI software.

3.1.3 Transformation into *V. cholerae*

The thereby confirmed plasmid was digested with NheI to linearize the DNA, which facilitates DNA uptake and ensures that no plasmid DNA would remain in the bacteria after introduction of the NheI-lacZ-kan-GFP-lacI-lacZ-NheI fragment into the *V. cholerae* genome by natural transformation. Natural competence of the *V. cholerae* strains A1552 wt and Δdns was induced through growth on chitin. The Δdns strain was used as a control because of its lack in a nuclease which leads to higher transformation frequencies compared to those of the wt. 2 µg of the respective DNA were added to both strains:

- VCXB21 chromosomal DNA to the positive controls
- lacZ-kan-lacZ fragment to the negative controls
- NheI-lacZ-kan-GFP-lacI-lacZ-NheI fragment to the actual samples

Transformants were screened on LB-Agar plates containing 75µg/ml of the antibiotic kanamycin and IPTG for the kan-GFP-lacI construct, as well as by blue white screening on LB-Agar plates supplemented with X-Gal. No conclusions could be drawn from the blue white screening as it was difficult to distinguish blue from white colonies. Transformation frequencies were estimated by dividing the transformed colony cell count of the antibiotic supplemented plates by the total cell count of the LB plates. Table 3 shows that the transformation frequency of the A1552 Δdns strain was about one order of magnitude higher than that of the wild type.

	LB	Kan75 +/- IPTG		transformation
	E-6	E0	E-2	frequency
A1552 wt / VCXB21	n/a	3;4	n/a	n/a
A1552 Δdns / VCXB21	3;8	lawn	50; n/a	9,091E-4
A1552 wt / lacZ-kan	11;13	178;137	1;0	1.313E-5
A1552 Δdns / lacZ-kan	7;7	>500	39;36	5.357E-4
A1552 wt / kan-GFP-lacI	9;11	3;8	0	5E-7
A1552 Δdns / kan-GFP-lacI	27;20	82;64	0	3.106E-6

Table 3. Transformation frequencies: Total cell count on LB plates. Naturally tra nsformed colonies counted on LB plates supplemented with kan and IPTG.

The eleven A1552 wt clones growing on LB plates supplemented with kanamycin and IPTG were screened for *gfp* by colony PCR optimized for *V. cholerae.* The *gfp* tagged A1552 wt strain was used as a positive control in the PCR reaction. As can be seen in Figure 15 all clones were successfully transformed with the NheI-lacZ-kan-GFP-lacI-lacZ-NheI fragment showing a band at ~700bp.

Figure 15. Agarose gel of transformed *V. cholerae* A1552 colony PCR products. L indicates the bands of the 1kb Plus DNA Ladder, numbers 1 to 11 represent the respective clones, number 12 the positive control.

Freezer stocks were made of ON LB cultures and stored at -80°C.

As preliminary experiments to determine the mutation frequency using the A1552 lacI strain revealed a surprisingly high frequency around 10E-6, the NheI-lacZ-kan-GFP-lacI-lacZ-NheI fragment was also transformed into the hapR complemented *V. cholerae* strain N16961 (Nielsen et al., 2006) to have a *V. cholerae* reference and thus rule out the possibility of A1552 being a mutator strain. The strain N16961 is hapR deficient. Among other genes, hapR is essential for natural competence in *V. cholerae*. Therefore, the hapR complemented mutant was used in subsequent experiments.
Natural transformation after competence induction through growth on chitin was performed according to the transformation of the A1552 strain.
Plate counts and calculated transformation frequency are listed in Table 4. A GFP specific colony PCR of the 5 colonies grown on LB plates supplemented with 75 µg/ml kanamycin and IPTG was performed resulting in a band at ~700bp if positive (see Figure 16). Freezer stocks of the positive clones were made and stored at -80°C.

	LB	Kan75 + IPTG	transformation frequency
A1552 wt / kan-GFP-lacI	2.5E8/ml	25/ml	1E-7

Table 4. Transformation frequency of the hapR complemented N16961 strain: Naturally transformed colonies counted on LB plates supplemented with kan and IPTG divided by the total cell count on LB plates.

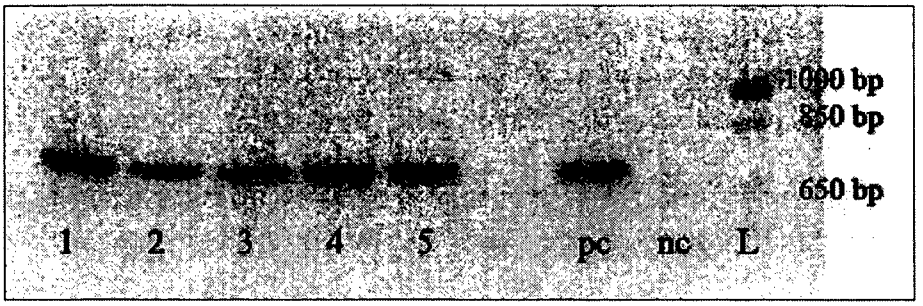

Figure 16. Agarose gel of transformed hapR complemented *V. cholerae* N16961 colony PCR products. L indicates the bands of the 1kb Plus DNA Ladder, numbers 1 to 11 represent the respective clones, number 12 the positive control.

3.2 Growth rates of planktonic *V. cholerae* cells

To demonstrate that the genetically engineered strains show the same growth phenotype as the respective wild type, growth rates of the following *V. cholerae* strains were determined:

- *V. cholerae* A1552 wt
- *V. cholerae* A1552 lacI
- *V. cholerae* N16961 hapR complemented wt
- *V. cholerae* N16961 hapR complemented lacI

Cultures of all strains mentioned above were grown in duplicate in 50 ml LB until they showed a constant OD_{600} value at 450 min, i.e. 7.5 h postinoculation. The averaged OD_{600} values of each time point and the corresponding standard deviations are listed in Table 5.

	t_0	t_1 = 45 min		t_2 = 90 min		t_3 = 135 min		t_4 = 180 min	
		x	σ	x	σ	x	σ	x	σ
A1552 wt	0.05	0.075	0.007	0.155	0.007	0.575	0.021	1.900	0.141
A1552 lacI	0.05	0.070	0.000	0.170	0.014	0.510	0.014	1.450	0.071
N16961 wt	0.05	0.165	0.007	0.385	0.007	0.845	0.021	2.250	0.071
N16961 lacI	0.05	0.155	0.007	0.370	0.000	0.810	0.014	2.300	0.283

	t_5 = 270 min		t_6 = 360 min		t_7 = 450 min	
	x	σ	x	σ	x	σ
A1552 wt	3.400	0.141	3.550	0.071	4.800	0.141
A1552 lacI	3.250	0.212	3.750	0.071	6.000	0.707
N16961 wt	3.500	0.000	4.100	0.566	5.350	0.636
N16961 lacI	3.550	0.071	4.200	0.141	6.000	0.141

Table 5. Growth curves of planktonic *V. cholerae* strains in LB at 30°C: OD_{600} values were measured at 0, 45, 90, 135, 180, 270, 360 and 450 min postinoculation. x indicates the averaged OD value, σ the standard deviation.

The growth curves resulting from these values are depicted in Figure 17. In Figure 18 the growth curves are represented using a logarithmic scale for the OD_{600} values.

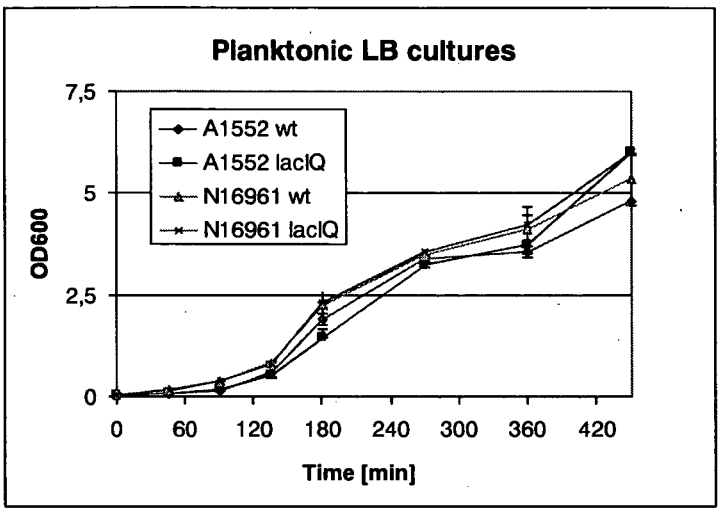

Figure 17. Growth curve of planktonic *V. cholerae* strains in LB at 30°C.

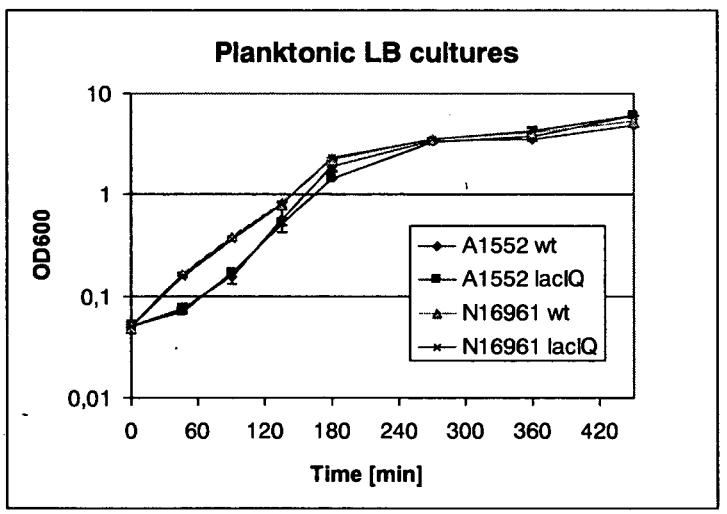

Figure 18. Logarithmic representation of growth curve data of planktonic *V. cholerae* strains in LB at 30°C

The growth rates of the respective strains were determined by linear regression of exponential phase data points (Table 6). The data confirms that the insertion of the kan-lacI-GFP construct in the lacZ gene does not cause an altered LB growth phenotype in the *V. cholerae* strains A1552 and N16961.

	μ [min^{-1}]	R^2
A1552 wt	0.0245	0.987
A1552 lacI	0.0226	0.9981
N16961 wt	0.0192	0.998
N16961 lacI	0.0197	0.9966

Table 6. Growth rates μ of *V. chlolerae* wt and lacI mutant strains A1552 and N16961 in LB.

By establishing growth curves of the A1552 wt and mutant strain in 25 ml 50 mM glycerol M9 MM, it was also shown that the genetically engineered *V. cholerae* strains' growth phenotypes do not differ from those of the respective wild type in M9 MM. Cultures were grown in triplicate for 30h until they reached stationary phase. Due to *V. cholerae*'s slow growth in M9 MM, it was sufficient to determine the OD_{600} every three hours. Unfortunately the 18 h time point was not successfully established. The averaged OD_{600} values of all other time points and the corresponding standard deviations are listed in Table 7, the growth curves resulting from these values are shown in Figure 19 using a logarithmic scale for the OD_{600} values.

	t_0	t_1 = 3 h		t_2 = 6 h		t_3 = 9 h		t_4 = 12 h		T_5 = 15 h	
		x	σ	x	σ	x	σ	x	σ	x	σ
A1552 wt	0.05	0.19	0.03	0.46	0.03	0.82	0.05	1.07	0.06	1.08	0.06
A1552 lacI	0.05	0.14	0.01	0.39	0.02	0.71	0.05	0.91	0.08	1.00	0.06

	t_6 = 18 h		t_7 = 21 h		t_8 = 24 h		t_9 = 27 h		t_{10} = 30 h	
	x	σ	x	σ	x	σ	x	σ	x	σ
A1552 wt	-	-	1.80	0.10	2.03	0.25	2.23	0.23	2.30	0.17
A1552 lacI	-	-	1.63	0.06	1.90	0.00	2.03	0.06	2.13	0.06

Table 7. Growth curves of planktonic *V. cholerae* strains in 50 mM glycerol M9 MM at 30°C: OD_{600} values measured every three hours postinoculation. x indicates the averaged OD values, σ the standard deviation.

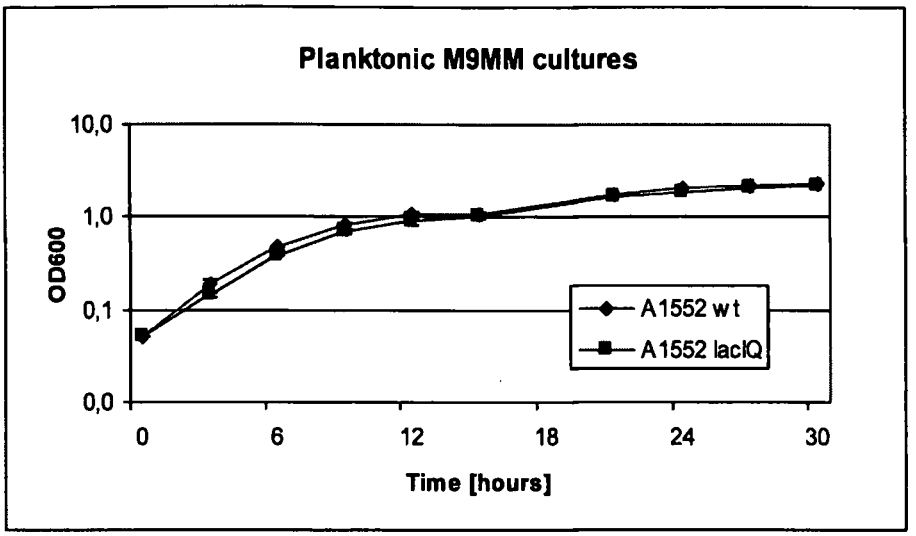

Figure 19. Logarithmic representation of growth curve data of planktonic *V. cholerae* strains in 50 mM glycerol M9 MM at 30°C

The growth rates of the A1552 wt and lacI mutant strain were determined by linear regression of exponential phase data points (Table 8). Corresponding to the observations in planktonic LB cultures, the data displays that the M9 MM growth phenotype does not change upon kan-lacI-GFP insertion in *lacZ*.

	μ [h^{-1}]	R^2
A1552 wt	0.3711	0.9889
A1552 lacI	0.3424	0.9998

Table 8. Growth rates μ of *V. chlolerae* wt and lacI mutant strains A1552 and N16961 in 50 mM glycerol M9 MM.

3.3 Mutation frequency in planktonic cultures

The mutation frequency of *V. cholerae* in planktonic cultures was determined both by analyzing the ratio of wt and mutated *V. cholerae* cells containing the kan-lacI-GFP mutation trap in *lacZ*, and by screening A1552 wt cells for spontaneous point mutations conferring antibiotic resistance.

3.3.1 Determination of the mutation frequency using the kan-lacI-GFP construct

First, *V. cholerae* A1552 cells containing the kan-lacI-GFP mutation trap in *lacZ* were grown in LB ,before plating in mid-log (OD_{600} ~ 0.4), late-log (OD_{600} ~ 1) and stationary

phase (OD_{600} ~ 5) and at 1 week postinoculation on both plain LB plates for the total cell count determination and on LB plates containing kan150 for the mutated cell count determination. Additionally, 50 mM glycerol M9 MM cultures were sampled, appropriately diluted and plated on plain LB plates and LB plates containing kan150 in log (OD_{600} ~ 0.4) and stationary phase (OD_{600} ~ 2), i.e. 6h and 24h postinoculation. The high antibiotic concentration of 150 µg/ml was used to eliminate non-mutated and thus non-fluorescing background colonies which were still growing on kan100 plates.
Subsequently the mutation frequency was calculated by dividing the mutated cell count by the total cell count. Both counts were averaged values from at least three independent experiments performed in duplicate (see Table 9).

	LB A1552 lacI		LB N16961 lacI		M9 A1552 lacI	
	x	σ	x	σ	x	σ
Mid-log phase	1.8E-06	8.6E-07	1.4E-06	7.2E-07	2.0E-06	4.2E-07
Late-log phase	1.3E-06	3.8E-07	2.1E-06	4.4E-07	-	-
Stationary phase	1.4E-06	1.9E-07	2.1E-06	4.3E-07	2.3E-06	3.1E-07
1 week old culture	3.7E-05	3.0E-05	7.0E-06	5.1E-06	-	-

Table 9. Mutation frequencies of the *V. cholerae* strains A1552 lacI and N16961 lacI in planktonic cultures in mid-log, late-log and stationary phase and at one week postinoculation. X indicates the value of the mutation frequency averaged over at least three independent experiments carried out in duplicate. σ shows the standard deviation.

The data is displayed in the bar charts in Figure 20 and Figure 21. The significance of the differences between the mutation frequencies of the respective phases are discussed in 4.2. Control samples were tested for fluorescence. Mutated cell colonies exhibited fluorescence of higher intensity than the GFP tagged A1552 wt used as a positive control.

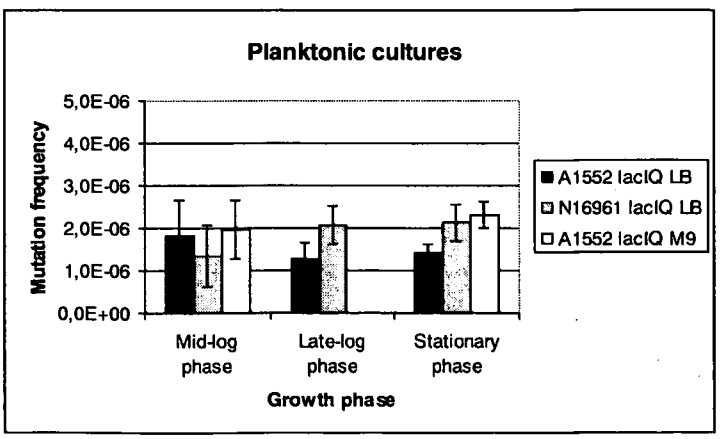

Figure 20. Comparison of the mutation frequencies of A1552 lacI and N16961 lacI determined for mid -log, late-log and stationary phase, in LB and 50 mM glycerol M9MM.

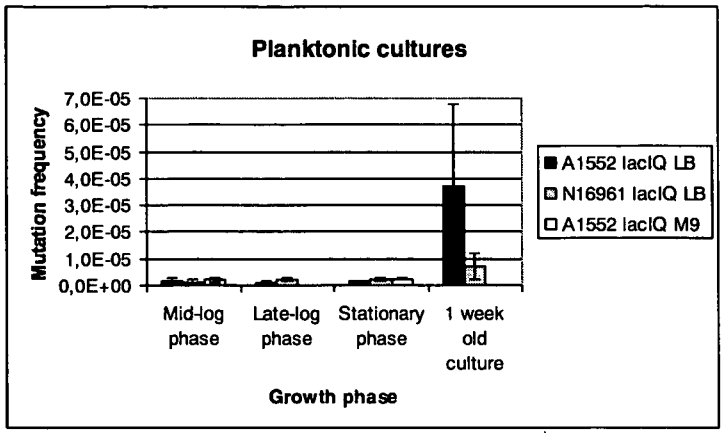

Figure 21. Comparison of the mutation frequencies of A1552 lacI and N16961 lacI d etermined for mid-log, late-log and stationary phase and one week postinoculation, in LB and 50 mM glycerol M9MM.

3.3.2 Determination of the mutation frequency through spontaneous antibiotic resistance

In order to obtain a second independent set of mutation frequency values, a series of experiments to examine the acquisition of spontaneous mutations conferring antibiotic resistance was started with the *V. cholerae* A1552 wt strain. The antibiotics used in these experiments were streptomycin, spectinomycin and nalidix acid, as single point mutations in the genes encoding these polypeptides can lead to antibiotic resistance in *V. cholerae*. Streptomycin was also used because the *V. cholerae* strain N16961 is resistant to this antibiotic.

As a first step, antibiotic concentrations that could be applied to the wild type *V. cholerae* strain A1552 were empirically determined as no suitable literature values were found. Therefore appropriately diluted cultures were plated on LB plates containing 25, 50 and 100 μg/ml streptomycin or spectinomycin and 5, 10 and 25 μg/ml nalidixic acid, respectively. The plates were inspected after ON incubation, little growth was observed and consequently the plates were left at 30°C for another 24h. After 48h of incubation at 30°C no colonies had grown on LB plates containing nalidixic acid. Therefore a lower antibiotic concentration, e.g. 2.5 μg/ml, should be used in further experiments. The determination of the optimal spectinomycin concentration proved to be more difficult as a lawn formed on plates containing 25 and 50 μg/ml, but no colonies grew on plates supplemented with 100 μg/ml of the antibiotic. No useful data can be concluded from the preliminary experiments regarding spectinomycin. For streptomycin 100 μg/ml seemed to be the optimal antibiotic concentration which is consistent with the fact that this concentration is used in *V. cholerae* strain N16961 cultures.

In order to determine the frequency of spontaneous mutations conferring streptomycin resistance in A1552 wt, mid-log phase LB cultures were diluted accordingly and plated on both plain LB plates for the total cell count determination and on LB plates supplemented with 100 µg/ml streptomycin for the mutated cell count determination. LB plates were incubated ON, plates containing the antibiotic for 48h.
The mutation frequency was calculated by dividing the number of cells on plates containing streptomycin, i.e. the mutated cells, by the total cell count. It averaged 1.5E-6 (data not shown).

3.4 Mutation frequency in biofilm cultures

3.4.1 Biofilm growth rate

Flow cells were harvested in 700 µl medium, i.e. the 200 µl media of the flow cell plus 500 µl LB medium; consequently the total cell count per ml has to be multiplied by 0.7 to calculate the total cell count per flow cell. Total cell counts per ml and per flow cell, respectively, are listed in Table 10.

	A1552 lacI		N16961 lacI	
	tcc / ml	tcc / flow cell	tcc / ml	tcc / flow cell
48h	9.3E+07	6.5E+07	2.9E+07	2.0E+07
60h	8.5E+08	5.9E+08	3.4E+08	2.4E+08
168h	1.4E+09	1.0E+09	1.7E+09	1.2E+09

Table 10. Total cell count of harvested biofilm cells per ml and per flow cell.

Using the total cell count (tcc) data an approximate biofilm growth curve was established (shown in Figure 22). The growth rates were estimated from the 48h and 60h timepoints.

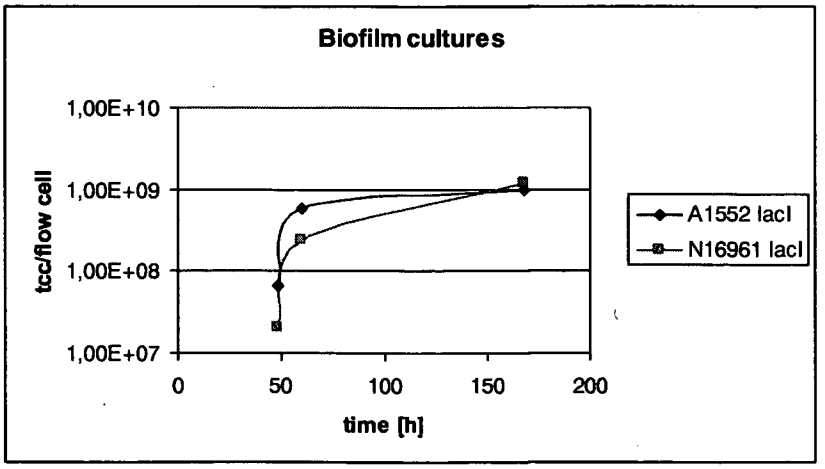

Figure 22. Logarithmic representation of growth curve data of V. cholerae biofilm cultures

	μ [h^{-1}]
A1552 lacI	0.1844
N16961 lacI	0.2051

Table 11. Biofilm growth rates μ of *V. chlolerae* lacI mutant strains A1552 and N16961.

3.4.2 Determination of the mutation frequency using the kan-lacI-GFP construct

Harvested biofilm cells were homogeneously suspended and diluted depending on the time point of harvesting, i.e. to the E-5 dilution for cells harvested at 48h post-inoculation and to the E-6 dilution for cells harvested at 60h and 168 h, respectively. 100 μl of the cell suspension were plated on both plain LB plates for the total cell count determination and LB plates containing kan150 for the mutated cell count determination. The mutation frequency of the biofilm cells was calculated by dividing the number of mutated cells by the total number of cells. The data is displayed in Table 12 and Figure 23.

	A1552 lacIQ		N16961 lacIQ	
	x	σ	x	σ
48h	2.3E-06	1.6E-06	1.4E-06	1.8E-06
60h	2.7E-06	3.4E-06	3.1E-07	2.8E-07
168h	2.9E-06	1.9E-06	2.2E-06	9.4E-07

Table 12. Mutation frequencies of *V. cholerae* A1552 lacI and N16961 lacI biofilm cultures 48, 60 and 168h postinoculation. X indicates the value of the mutation frequency averaged over three independent experiments carried out in duplicate. σ shows the standard deviation.

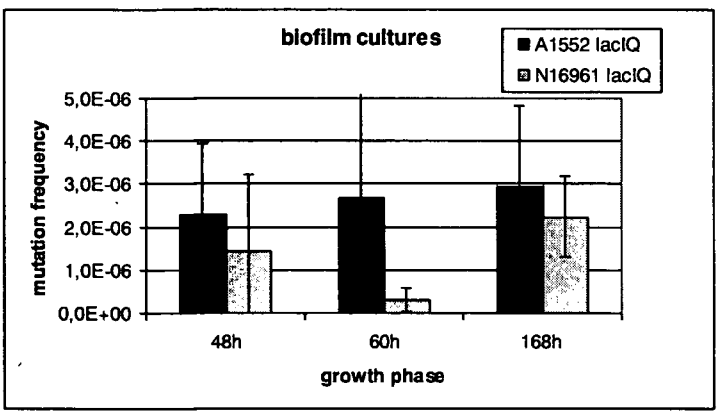

Figure 23. Comparison of the mutation frequencies of A1552 lacI and N16961 lacI biofilm cultures determined at 48, 60 and 168h postinoculation.

The significance of the differences between the mutation frequencies of the respective time points as well as the high standard deviations are discussed in 4.3. Control samples were tested for fluorescence.

3.4.3 CLSM images of biofilm cultures

Biofilm cells were first checked at 24h postinoculation. As no significant growth had happened to this point, cultures were left to grow another 24h and then were stained and imaged at 48, 60 and 168h postinoculation. Cells were stained with the cell-permeant SYTO63 red fluorescent nucleic acid stain (Invitrogen) to visualize the total biofilm. Non-mutant A1552 lacI cells with the previously developed reporter would thus be red when scanning for Syto63 with the He/Ne laser at 654 nm, whereas mutated cells would appear green when scanning for GFP with the Ar/ArKr laser at 488 nm and yellow when scanning for GFP and Syto63 simultaneously.

Imaging at 48h showed that an A1552 lacI biofilm had started to form on the flow channel surface, but was not yet covering the entire surface. The N16961 lacI strain was slower in biofilm formation, leaving wide parts of the surface free. Mushrooms, i.e. spherical structures, are visible for A1552 lacI, but not for N16961. No mutated cells (GFP positive) were found in both biofilms despite extensive scanning. CLSM images of the A1552 lacI and N16961 lacI are shown in Figure 24 and Figure 25.

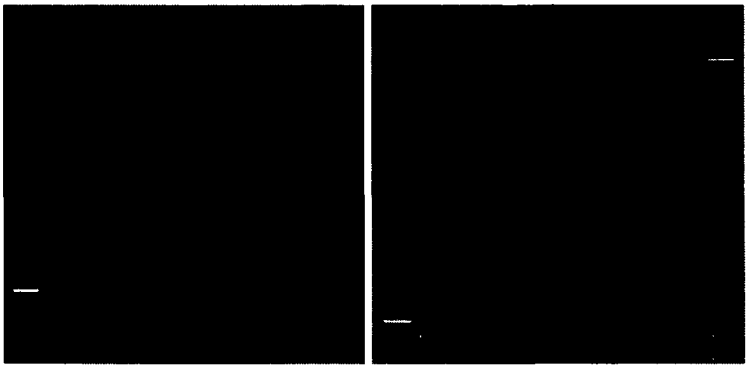

Figure 24. CLSM images of the 48h *V. cholerae* A1552 lacI biofilm grown in a hydrodynamic flow chamber amended with 1.5mM glycerol M9 MM. He/Ne at 654/675 nm. Scale bars are 20 µm.

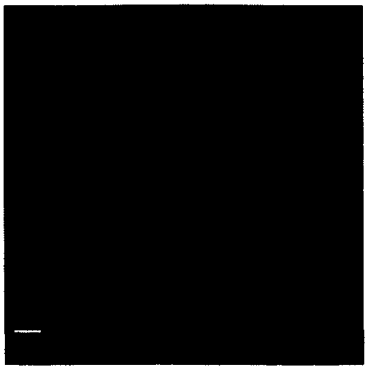

Figure 25. CLSM image of the 48h *V. cholerae* N16961 lacI biofilm grown in a hydrodynamic flow chamber amended with 1.5mM glycerol M9 MM. He/Ne at 654/675 nm. Scale bar indicates 20 μm.

At 60h postinoculation, most of the side of the flow channel surface was covered by a 10 μm thick, flat A1552 lacI biofilm while the central section contained mushrooms up to 60 μm thick. After thorough scanning, isolated mutated cells were found in the thinner areas of the biofilm. Figure 26 shows the simultaneous excitation of Syto63 in all biofilm cells and GFP in the mutated cells on the left. A1552 lacI cells are shown in red and mutated cells in yellow. The image in the middle is a digitally processed version of the one to the left. To facilitate visualization of the mutated cells the blend opacity setting has been applied, resulting in more transparent biofilm layers. The image on the right shows the mutated cells of the biofilm upon excitation with the Ar/ArKr laser only.

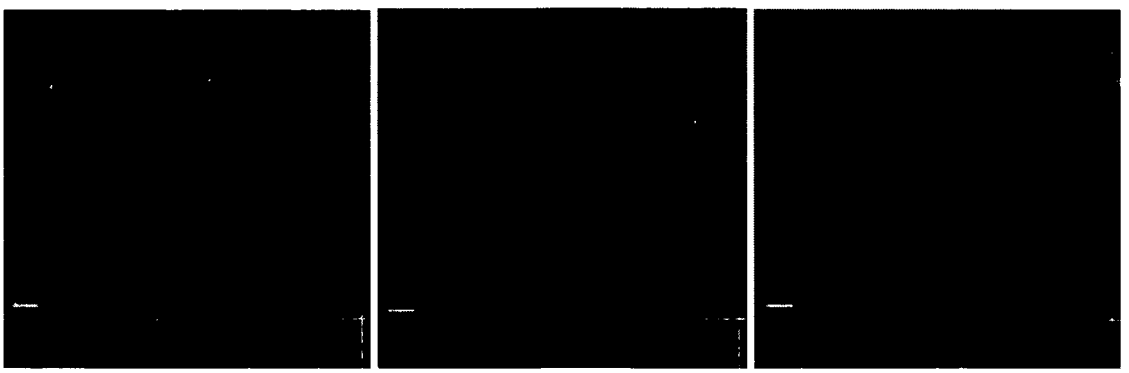

Figure 26. CLSM images of the 60h *V. cholerae* A1552 lacI biofilm grown in a hydrodynamic flow chamber amended with 1.5mM glycerol M9 MM. He/Ne at 654/675 nm. Ar/ArKr at 488/550 nm. Scale bars are 20 μm. a) Simultaneous GFP and Syto63 excitation: total biofilm in red, mutated cells in yellow b) Simultaneous GFP and Syto63 excitation, using the blend opacity setting for image processing for better visualization of the mutated cells c) GFP excitation only: mutated cells in green.

At the 168h timepoint, that is one week postinoculation, the original biofilm had gone through a detachment phase after which a full, three-dimensional biofilm redeveloped. The post-detachment biofilm was more homogenous, characterized by a flatter structure of

~10μm thickness. Figure 27 displays the Syto63 stained A1552 lacI biofilm at 168h postinoculation. The left image shows the scan upon simultaneous GFP and Syto63 excitation, using the blend opacity setting for image processing. Again, the total biofilm appears in red, mutated cells in green. Mutated cells only are visible after GFP excitation in the right image. Figure 28 shows the image of the 168h N16961 lacI biofilm upon simultaneous GFP and Syto63 excitation. Despite of the extensive searching no mutated cells were found in the biofilm for this strain, in contrast to the case with the A1552 lacI.

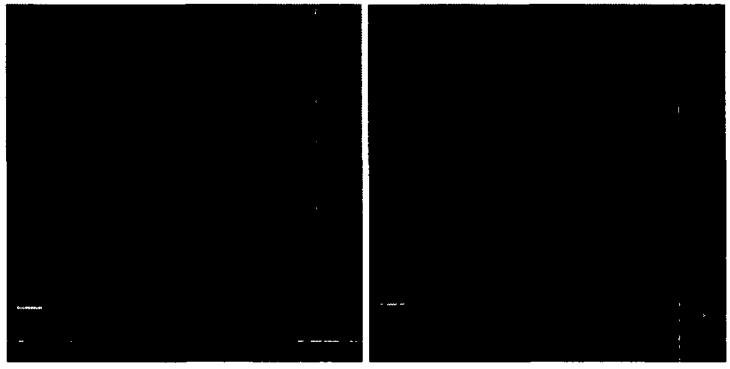

Figure 27. CLSM images of the 168h *V. cholerae* A1552 lacI biofilm grown in a hydrodynamic flow chamber amended with 1.5mM glycerol M9 MM. He/Ne at 654/675 nm. Ar/ArKr at 488/550 nm. Scale bars indicate 20 μm. a) Simultaneous GFP and Syto63 excitation, using the blend opacity setting for image processing: total biofilm in red, mutated cells in green b) GFP excitation only: mutated cells in green.

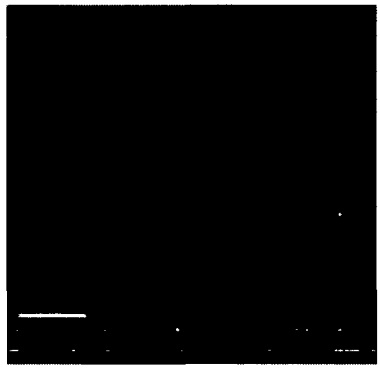

Figure 28. CLSM image of the 168h *V. cholerae* N16961 lacI biofilm grown in a hydrodynamic flow chamber amended with 1.5mM glycerol M9 MM. Simultaneous GFP and Syto63 excitation. He/Ne at 654/675 nm. Ar/ArKr at 488/550 nm. Scale bars indicate 50 μm.

4 Discussion

4.1 Natural transformation

Transformation upon induction of natural competence through growth on chitin proved to be a suitable novel genetic engineering technique for *V. cholerae,* with the following advantages over the Tn7-based delivery system: the five day natural transformation procedure is faster than the latter by one day and more straightforward in manipulating. Moreover, after construction of a vector harbouring appropriate flanking regions and restriction sites the construct can be introduced in different *V. cholerae* strains, i.e. A1552 and N16961, without any further molecular work. The transformation frequency ranging from 5E-7 in *V. cholerae* strain A1552 to E-7 in strain N16961 was adequate for the presented experiments, but two magnitudes smaller than the transformation frequency shown for *V. cholerae* strain A1552 by Meibom et al., 2005. All cells growing on plates supplemented with a selective antibiotic were positive clones.

4.2 Mutation frequency in planktonic cultures

Both the A1552 lacI and the N16961 lacI *V. cholerae* strains showed a mutation frequency of one to two mutated cells per one million cells in log and stationary phase after planktonic cultivation in LB and M9MM medium, respectively. The differences between these two growth phases are insignificant. After one week of cultivation the A1552 lacI strain exhibited a tenfold higher mutation frequency of three to four cells per 100,000 cells, the N16961 lacI mutation frequency tripled resulting in seven cells per one million cells. This higher frequency could be explained by the fact that the mutation frequency is not a constant value as is the mutation rate, but a relative value that increases as cell counts multiply and thus mutants accumulate. The rise in mutation frequency could also be a response of the bacteria to the environmental stress posed by the nutrient limitation after one week of batch cultivation. Under the selective pressure of starvation the regulated phenomenon of stress-enhanced bacterial mutation has been observed: bacterial populations are able to adapt rapidly to their environment by generating more diversity through two mechanisms, i.e. through mutator strains that exhibit a higher mutation rate than wt strains (Miller, 1996; Mao et al., 1997) and through low-fidelity DNA polymerases that introduce genetic variation (Radman, 1999). In the case of both mechanisms a mutation frequency higher than the expected spontaneous mutation frequency will be observed under starvation conditions as a result of stress-induced mutagenesis, a phenomenon also referred to as "adaptive mutation" (Roth et al., 2006).

The occurrence of higher spontaneous mutation frequencies in stationary phase as an attempt to overcome stress has been elucidated previously for mutations conferring rifampicin resistance and novobiocin resistance in the *V. cholerae* strains O395 and CM319 (Banerjee and Chowdhury, 2006) and is consistent with my data regarding the difference between exponential and stationary phase frequencies. The absolute values, however, differing by three orders of magnitude, should not be compared directly because of the disparity in the mutation loci.

In the presented mutation frequency experiments the genetic construct used as a mutation trap led to kanamycin resistant, GFP expressing cells via a mutation in either the lacoperator or *lacI* gene. The standard deviations of the mutation frequencies determined from the data of three independent experiments each carried out in triplicates are listed in Table 9 and illustrated in Figure 21. They seem extremely high ranging from about 10% in log and stationary phase to almost 100% in the one week old cultures. According to Luria and Delbrück's mutation hypothesis, which stands in contrast to the hypothesis of acquired immunity, the number of mutated bacteria depends on the time of occurrence of the mutation: Mutation events taking place early during the growth of a population have vast consequences. The mutation frequency is therefore distributed with an abnormally high variance as early mutations will lead to a higher frequency than later occurring ones (Luria and Delbrück, 1943). Thus, the high standard deviation in the obtained mutation frequency data is consistent with Luria and Delbrück's fluctuation experiment. Certainly, the lacI construct proved to be a successful tool for the determination of mutation frequencies in both A1552 and N16961 *V. cholerae* planktonic cultures resulting in a mutation frequency of approximately one mutated cell in a million cells.

To obtain a second reference value for random mutations in another locus, streptomycin was used in the next experiment. This aminoglycoside antibiotic inhibits bacterial growth by targeting the biosynthesis of proteins through direct interaction with the small ribosomal subunit, more specifically with the ribosomal protein S12 (Carter et al 2000). It has been shown that a number of mutations in the *rpsL* gene encoding this polypeptide can cause streptomycin resistance (Wittmann and Apirion 1975; Timms et al, 1992; Finken et al, 1993). A second mutation frequency value for planktonic *V. cholerae* cells could thus be determined via spontaneous mutations conferring streptomycin resistance by simply plating cultures on streptomycin-containing plates. The calculated frequency averaged at one to two mutated cells per one million cells. This value corresponds to the data established by use of the lacI construct when regarding mutations per gene. However, a comparison of these frequencies is not that straight-forward as the molecular basis leading

to the different mutants has to be taken into consideration. Whereas mutations in the lac operator and promoter as well as the gene encoding the lac repressor are able to generate kanamycin resistance in the later, only mutations in the *rpsL* gene account for streptomycin resistance. For a more conclusive comparison between the frequencies of these distinct mechanisms more quantitative molecular data would be needed. For example, the gene of interest of a representative sample of mutants could be sequenced. This procedure surpassed the scope of my work though.

The spontaneous mutation frequency conferring streptomycin resistance determined for *V. cholerae* A1552 lacI is comparable to that in *Yersinia pestis* which averages at 10^{-6} (Louie et al., 2007). It is also similar to the frequency of spontaneous mutations conferring resistance to the aminoglycoside spectinomycin in *Chlamydia psittaci* (Binet and Maurelli, 2005). This remarkable similarity among the spontaneous mutation rates of different microbes has been observed previously and can be explained by the fact that an optimized mutation rate has evolved (Drake, 1991).

There was little growth observed on plates containing streptomycin after ON incubation at 30°C and colonies were thus counted 48h post-plating. This slow growth of drug-resistant bacteria compared to susceptible bacteria is termed the "cost of resistance" phenomenon, originating from the fact that mutations that confer resistance also reduce the organism's overall fitness (Andersson and Levin, 1999).

4.3 Mutation frequency in biofilm cultures

As shown in Table 12 and Figure 23, respectively, the biofilm cultures of the *V. cholerae* strain A1552 lacI showed a constant mutation frequency of two to three mutated cells per one million cells at 48h, 60h and 168h post-inoculation. The mutation frequency determined for the N16961 lacI strain was lower and more variable, amounting to one to two mutated cells per one million cells at 48h and 168h post-inoculation, and three mutated cells per ten million cells after 60h of biofilm cultivation. Again, this inconsistency and the high standard deviation of all biofilm experiments can be explained by Luria and Delbrück's mutation hypothesis (Luria and Delbrück, 1943). The mutation frequency value for N16961 lacI at 60h postinoculation seems to be an outlier.
Imaging of the biofilm cultures with a confocal laser scanning microscope allowed the real-time observation of evolutionary processes, i.e. the time and place of occurrence of lacI mutated cells in the biofilm could be visualized. At 48h post-inoculation an A1552 lacI biofilm containing spherical structures had started to form on the flow channel surface, but was not covering the entire surface yet. Biofilm formation of the N16961 lacI strain

was slower, leaving wide parts of the surface free. The total cell count of the flow cell at 48h averaged about sixty million cells for the A1552 lacI strain and approximately thirty million cells for the N16961 lacI strain. This explains why no mutated cells were found in both biofilms despite extensive scanning, as merely one hundred mutated cells are expected to be present in the flow cell. At 60h post-inoculation mutated cells were spotted in the 10 μm thick, flat A1552 lacI biofilm covering most of the side of the flow channel surface, but not in any of the mushrooms as shown in Figure 26. 600 million cells are present in the biofilm at this timepoint, about 1000 of them are expected to have mutated in the lacI construct. In the flatter and more homogeneous post-detachment biofilm at 168h post-inoculation the cell populations have doubled and average above one billion cells per flow cell. For the A1552 lacI strain mutated cells were imaged as can be seen in Figure 27, for N16961 no cells expressing GFP were found despite thorough scanning and the fact that approximately 1000 mutated cells were present in the biofilm. Reasons for this lack of success might lie in the fact that N16961 forms different, i.e. less pronounced biofilms than A1552, and that even more scanning would have been necessary to localize mutated cells.

4.4 Comparison

Although the determined values for the mutation frequency in planktonic and biofilm cells are within the same order of magnitude a direct comparison would oversimplify the issue. Mutation frequencies are dependent on the amount of cell doublings that have occurred and should therefore only be compared if growth rates are equivalent. The biofilm growth rate was estimated from merely two time points, i.e. at 48h and 60h post-inoculation, linear regression could not be applied and no data was collected at early time points. Thus there is no proof that the estimated growth rate is representing the exponential phase of the biofilm culture. Moreover, in the estimated growth rate, detached cells are not accounted for. In future experiments the exponential growth rate should be determined as has been done for the planktonic growth rate, taking the cells in the outflow into consideration.

A challenge remaining is the heterogeneity of the biofilm in which bacterial growth activity is distributed (Sternberg et al., 1999). Growth and mutation frequencies, respectively, will not be the same in the overall biofilm and working with averaged values might not account for the complexity of the biofilm.

While posing several challenges, a determination of the mutation rate instead of the mutation frequency would be more conclusive. First, it is a more accurate and biologically significant reflection of underlying mutagenic mechanisms when properly determined. Second, it allows direct comparison of planktonic and biofilm cultures. A number of methods to estimate mutation rates have been developed for specific experimental conditions. For example the mutant accumulation method or a fluctuation analysis could be

used to determine the mutation rate of *V. cholerae* planktonic and biofilm cultures (Foster, 2006).

The results of this study provide us with precise values for the mutation frequencies of *V. cholerae* strains A1552 and N16961, and thus give insight into the evolutionary mechanisms contributing to the diversity of biofilm cells. These findings are likely to contribute to our understanding of biofilm stability and might therefore have implications for both industrially as well as medically relevant biofilms. In addition, resistance of biofilm associated microorganisms to antibiotics and chemicals is a major concern and not yet well understood. Elucidating the evolutionary fate of cells in a biofilm community is crucial for understanding the dynamics of biofilms.

5 References

Journal Articles:

Andersson, D. I., Levin B. R., The biological cost of antibiotic resistance. *Curr Opin Microbio* **1999**, 2:489-493.

Andersen J.B., Sternberg C., Poulsen L.K., Bjorn S.P., Givskov M., Molin S. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **1998,** 64: 2240–2246.

Anwar H., Strap J.L., Costerton J.W. Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrob Agents Chemother* **1992**, 36: 1347 - 1351.

Banerjee S., Chowdhury R., An orphan DNA (cytosine-5-)-methyltransferase in Vibrio cholerae. Microbiology **2006**, 152: 1055-1062.

Beech I.B., Sunner J., Biocorrosion: towards understanding interactions between biofilms and metals. *Curr Opin Biotechnol* **2004**, 15(3):181-6.

Beyhan S., Bilecen K., Salama S.R., Casper-Lindley C., Yildiz F.H., Regulation of rugosity and biofilm formation in *Vibrio cholerae*: comparison of VpsT and VpsR regulons and epistasis analysis of vpsT, vpsR, and hapR. *J Bacteriol* **2007**, 189(2):388-402.

Binet R., Maurelli A.T., Frequency of spontaneous mutations that confer antibiotic resistance in *Chlamydia* spp. *Antimicrob Agents Chemother* **2005**, 2865-2873.

Brooun A., Liu S., Lewis K., A Dose-Response Study of Antibiotic Resistance in *Pseudomonas aeruginosa* Biofilms. *Antimicrob Agents Chemother* **2000,** 44: 640 - 646.

Butler SM, Camilli A., Going against the grain: chemotaxis and infection in Vibrio cholerae. *Nat Rev Microbiol* **2005**, 3(8):611-20.

Carter, A. P., Clemons W. M., Brodersen D. E., Morgan-Warren R. J., Wimberly B. T., Ramakrishnan V., Functional insights from the structure of the 30S ribosomal subunit and its interaction with antibiotics. *Nature* **2000**, 407:340-348

Christensen B.B., Sternberg C., Andersen J.B., Palmer R.J., Nielsen A.T., Giskov M., Molin S., Molecular tools for study of biofilm physiology. *Methods Enzymol* **1999**, 310:20–42.

Colwell R.R., Global climate and infectious disease: the cholera paradigm. *Science* **1996**, 20;274(5295):2025-31.

Davies D.G., Chakrabarty A.M., Geesey G.G., Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **1993**, 59(4):1181-6.

Davies D.G., Geesey G.G., Regulation of the alginate biosynthesis gene algC in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Appl Environ Microbiol* **1995**, 61(3):860-7.

Drake J.W., A constant rate of spontaneous mutation in DNA-based microbes. *Proc Natl Acad Sci U S A* **1991**, 15;88(16):7160-4.

Espeland E. M., Wetzel R. G., Complexation, stabilization, and UV photolysis of extracellular and surface-bound glucosidase and alkaline phosphatase: implications for biofilm microbiota. *Microb Ecol* **2001**, 42:572–585.

Finken, M., Kirschner P., Meier A., Böttger E. C., Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Mol Microbiol* **1993**, 9:1239-1246.

Foster P.L.., Methods for determining spontaneous mutation rates. *Methods Enzymol* **2006**, 409:195-213.

Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. *Trends Microbiol* **2005**, 13(1):34-40.

Hanahan D., Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **1983**, 166(4):557-80.

Handke, L.D., Conlon K.M., Slater S.R., Elbaruni S., Fitzpatrick F., Humphreys H., Giles W.P., Rupp M.E., Fey P.D., O'Gara J.P., Genetic and phenotypic analysis of biofilm phenotypic variation in multiple *Staphylococcus epidermidis* isolates. *J Med Microbiol* **2004**, 53(5):367-74.

Heidelberg J.F., Eisen J.A., Nelson W.C., Clayton R.A., Gwinn M.L., Dodson R.J., Haft D.H., Hickey E.K., Peterson J.D., Umayam L., DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **2000**, 406:477–483.

Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR., Ecological relationships between Vibrio cholerae and planktonic crustacean copepods. *Appl Environ Microbiol* **1983**, 45(1):275-83.

Inoue H., Nojima H., Okayama H., High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **1990**, 96(1):23-8.

Kaper J.B., Morris, J.G., Jr, Levine, M.M., Cholera. *Clin Microbiol Rev* **1995**, 8: 48-86

Kirisits M.J., Prost L., Starkey M., Parsek M.R., Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **2005**, 71(8):4809-21.

Klausen M., Gjermansen M., Kreft J.U., Tolker-Nielsen T., Dynamics of development and dispersal in sessile microbial communities: examples from

Pseudomonas aeruginosa and *Pseudomonas putida* model biofilms. *FEMS Microbiol Lett* **2006**, 261(1):1-11.

Koh K.S., Lam K.W., Alhede M., Queck S.Y., Labbate M., Kjelleberg S., Rice S.A., Phenotypic diversification and adaptation of *Serratia marcescens* MG1 biofilm-derived morphotypes. *J Bacteriol* **2007**, 189(1):119-30.

Lambertsen L., Sternberg C. & Molin S., Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ Microbiol* **2004**, 6: 726–732.

Leid J. G., Shirtliff M. E., Costerton J. W., Stoodley, P. Human leukocytes adhere, penetrate, and respond to *Staphylococcus aureus* biofilms. *Infect Immun* **2002**, 70:6339–6345.

Le Magrex-Debar E., Lemoine J., Gelle M. P., Jacquelin L. F., Choisy C., Evaluation of biohazards in dehydrated biofilms on foodstuff packaging. *Int J Food Microbiol* **2000**, 55: 239–234.

Lim B., Beyhan S., Yildiz F.H., Regulation of Vibrio polysaccharide synthesis and virulence factor production by CdgC, a GGDEF-EAL domain protein, in *Vibrio cholerae*. *J Bacteriol* **2007**, 189(3):717-29.

Liu Z., Stirling F.R., Zhu J., Temporal quorum-sensing induction regulates *Vibrio cholerae* biofilm architecture. *Infect Immun* **2007**, 75(1):122-6.

Louie A., Deziel M.R., Liu W., Drusano G.L., Impact of resistance selection and mutant growth fitness on the relative efficacies of streptomycin and levofloxacin for plague therapy. *Antimicrob Agents Chemother* **2007**, 51(8):2661-7

Luria S.E., Delbrück M., Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics* **1943**, 28(6):491-511.

Mah T.F., O'Toole G.A., Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **2001**, 9(1):34-9.

Mao E.F., Lane L., Lee J., Miller J.H., Proliferation of mutators in a cell population. *J Bacteriol* **1997**, 179(2):417-22.

McNeill K., Hamilton I. R., Acid tolerance response of biofilm cells of *Streptococcus mutans*. *FEMS Microbiol Lett* **2003**, 221:25–30.

Meibom K.L., Blokesch M., Dolganov N.A., Wu C.Y., Schoolnik G.K., Chitin induces natural competence in *Vibrio cholerae*. *Science* **2005**, 310 (5755):1824-7.

Miller, J. H., Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. *Annu Rev Microbiol* **1996**, 50:625-643.

Miller, V. L., Mekalanos J. J., A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **1988**, 170:2575–2583.

Monk I.R., Cook G.M., Monk B.C., Bremer P.J., Morphotypic conversion in *Listeria monocytogenes* biofilm formation: biological significance of rough colony isolates. *Appl Environ Microbiol* **2004**, 70(11):6686-94.

Morris J.G. Jr., Cholera and other types of vibriosis: a story of human pandemics and oysters on the half shell. *Clin Infect Dis* **2003**, 15;37(2):272-80.

Mueller R.S., McDougald D., Cusumano D., Sodhi N., Kjelleberg S., Azam F., Bartlett D.H., *Vibrio cholerae* strains possess multiple strategies for abiotic and biotic surface colonization. *J Bacteriol* **2007**, 189(14):5348-60.

Müller J., Miller M.C., Nielsen A.T., Schoolnik G.K., Spormann A.M. vpsA- and luxO-independent biofilms of *Vibrio cholerae*. *FEMS Microbiol Lett* **2007**, 275(2):199-206.

Ngwai Y.B., Adachi Y., Ogawa Y., Hara H., Characterization of biofilm-forming abilities of antibiotic-resistant *Salmonella typhimurium* DT104 on hydrophobic abiotic surfaces. *J Microbiol Immunol Infect* **2006**, 39(4):278-91.

Nicolella C., van Loosdrecht M.C., Heijnen J.J., Wastewater treatment with particulate biofilm reactors. *J Biotechnol* **2000**, 9;80(1):1-33.

Nielsen A.T., Dolganov N.A., Otto G., Miller M.C., Wu C.Y., Schoolnik G.K., RpoS controls the *Vibrio cholerae* mucosal escape response. *PLoS Pathog* **2006**, 2(10):e109.

Prigent-Combaret C., Vidal O., Dorel C., Lejeune P. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *J Bacteriol* **1999**, 181(19):5993-6002.

O'Gara J.P., Humphreys H., *Staphylococcus epidermidis* biofilms: importance and implications. *J Med Microbiol* **2001**, 50(7):582-7.

OToole G, Kaplan HB, Kolter R., Biofilm formation as microbial development. *Annu Rev Microbiol* **2000**, 54:49-79.

OToole G.A., Kolter R., Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **1998**, 28(3):449-61.

Pratt L.A., Kolter R., Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* **1998**, 30(2):285-93.

Prigent-Combaret C., Vidal O., Dorel C., Lejeune P., Abiotic Surface Sensing and Biofilm-Dependent Regulation of Gene Expression in *Escherichia coli*. *J Bacteriol* **1999**, 181 (19): 5993-6002.

Radman, M., Enzymes of evolutionary change. *Nature* **1999**, 401:866-887.

Rainey P.B., Travisano M., Adaptive radiation in a heterogeneous environment. *Nature* **1998**. 394(6688):69-72

Rainey P.B., Buckling A., Kassen R., Travisano M. The emergence and maintenance of diversity: insights from experimental bacterial populations. Trends *Ecol Evol* **2000**, 15(6):243-247.

Reisner A., Haagensen J.A., Schembri M.A., Zechner E.L., Molin S., Development and maturation of *Escherichia coli* K-12 biofilms. *Mol Microbiol* **2003**, 48(4):933-46.

Roth J.R., Kugelberg E., Reams A.B., Kofoid E., Andersson D.I. Origin of mutations under selection: The adaptive mutation controversy. *Annu Rev Microbiol* **2006**, 60, 477-501

Simon, R., Priefer U., Puehler A., A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Biotechnology* **1983**, 1:784–791.

Singh R., Paul D., Jain R.K., Biofilms: implications in bioremediation. *Trends Microbiol* **2006**, 14(9):389-97.

Sternberg C., Christensen B.B., Johansen T., Nielsen A.T., Andersen J.B., Givskov M., Molin S., Distribution of bacterial growth activity in flow-chamber biofilms. *Appl Environ Microbiol* **1999**, 65(9):4108-17.

Stewart P.S., Costerton J.W., Antibiotic resistance of bacteria in biofilms. *Lancet* **2001**, 358, 135–138.

Teitzel G. M., Parsek M. R., Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **2003**, 69:2313–2320.

Thormann K.M., Duttler S., Saville R.M., Hyodo M., Shukla S., Hayakawa Y., Spormann A.M. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. *J Bacteriol* **2006**, 188(7):2681-91.

Thormann K.M., Saville R.M., Shukla S., Pelletier D.A., Spormann A.M. Initial Phases of biofilm formation in *Shewanella oneidensis* MR-1. *J Bacteriol* **2004**, 186 (23): 8096-104.

Thormann K.M., Saville R.M., Shukla S., Spormann A.M. Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. *J Bacteriol* **2005**, 187(3):1014-21.

Timms, A.R., Steingrimsdottir H., Lehmann A. R., Bridges R. A., Mutant sequences in the *rpsL* gene of *Escherichia coli* B/r: mechanistic implications for spontaneous and ultraviolet light mutagenesis. *Mol Gen Genet* **1992**, 232:89-96.

Watnick P.I., Kolter R., Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol Microbiol* **1999**, 34(3):586-95.

Wittmann, H. G., Apirion D., Analysis of ribosomal proteins in streptomycin resistant and dependent mutants isolated from streptomycin independent *Escherichia coli* strains. *Mol Gen Genet* **1975**, 141:331-341.

Xu, H.-S., Roberts N., Singleton F.L., Attwell R.W., Grimes D.J., Colwell and R.R., Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb Ecol* **1982**, 8:313-323.

Yildiz F.H., Schoolnik G.K., Role of rpoS in stress survival and virulence of *Vibrio cholerae*. *J Bacteriol* **1998**, 180(4):773-84.

Zobell C.E., The Effect of Solid Surfaces upon Bacterial Activity. *J Bacteriol* **1943**, 46(1):39-56.

Books:

Sambrook J., Russell D.W., *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, USA, 2001.

Book Sections:

Hansen S.K., Molin S., Temporal Segregation: Succession in Biofilms. In *Microbial Evolution*, Eds. Miller R.V. and Day M.J. 192-213

Lewin, B., Genes are DNA. In *Genes* IX. Jones & Bartlett Publishers: Sudbury, MA, USA, 2007.

Russell, P.J., Gene Mutation. In *Genetics*, 5th ed., The Benjamin/Cummings Publishing Company, Inc: Menlo Park, CA, USA, 1998, (615 -648).

Ryan K.J., *Vibrio*, *Campylobacter* and *Helicobacter*. In *Sherris Medical Microbiology*, 4th ed., McGraw Hill, 2004, (376–7) Ray C.G and Ryan K.J.